MITOCHONDRIAL BIOENERGETICS: AN INTEGRATED PLATFORM TO STUDY INTERACTIONS OF MULTIPLE STRESSORS

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Ravinder Sappal

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ABSTRACT

Animals must expend energy to deal with a wide array of stressors associated with environmental change. Because mitochondria produce >90% of the energy requirement of the cell, they are likely the fundamental drivers of responses to environmental change. The primary goal of my thesis was to explore the interactions of three common stressors in aquatic systems – temperature, metals (copper: Cu) and hypoxia– on mitochondrial bioenergetics. To achieve this, I first performed a series of *in vitro* experiments using rainbow trout, *Oncorhynchus mykiss* liver mitochondria energized with complex I and II substrates to characterize the acute interactive responses of temperature and Cu on mitochondrial function. These studies revealed that Cu altered the basal and maximal mitochondrial oxidation rates differently depending on the metal dose and temperature. Mechanistically, I showed that Cu impairs oxidative phosphorylation in part by inhibiting the electron transport system (ETS) enzymes, stimulating proton leak, inducing mitochondrial permeability transition pore and dissipating inner membrane potential. Importantly, temperature exacerbated the effects of Cu suggesting that environmental warming, e.g., due to climate change, may sensitize fish to Cu toxicity.

The next study combined *in vitro* and *in vivo* approaches to shed light on how persistent elevated temperature (warm acclimation) modulates the effects of acute temperature increase, hypoxia-reoxygenation (HRO) and/or Cu on mitochondrial function. Sequential inhibition and activation of mitochondrial ETS enzyme complexes permitted the measurement of respiratory activities supported by ETS complexes I-IV in one run and allowed me to identify segments/components of the ETS that are resilient or susceptible to single and combined effects of temperature, Cu and HRO. This study also revealed that warm acclimation blunted the sensitivity of the ETS to acute temperature rise and, together with HRO, sensitized the ETS to Cu.

My fourth study examined how warm acclimation influences the ability of fish to handle individual and joint effects of subsequent acute temperature shifts, hypoxia and Cu stress by exposing fish *in vivo* to the three stressors. Here I measured mitochondrial oxidation and apical endpoints indicative of stress and organismal energy status to assess the relevance of energy metabolism endpoints *in vivo*. I showed that warm acclimation reduced fish condition, promoted anaerobic metabolism, decelerated the ETS and altered the responses of fish to acute temperature shifts, hypoxia and Cu. Moreover, Cu and hypoxia showed reciprocal antagonistic interaction on the ETS and plasma metabolites, with modest additive actions limited to proton leak.

The final study highlighted the functional-biochemical and transcriptional responses of fish to warm acclimation and short-term exposures to Cu and hypoxia. In this *in vivo* study, activities of ETS enzyme complexes and targeted analyses of transcripts encoding for proteins involved in mitochondrial oxidation, metals detoxification/stress response and energy sensing were done in isolated liver mitochondria and in whole liver and gill tissues by RT-qPCR. Warm acclimation inhibited activities of ETS enzymes while effects of Cu and hypoxia depended on the enzyme and thermal acclimation status. The genes encoding for proteins involved in mitochondrial oxidation, metals detoxification/stress response and energy sensing were all strongly regulated by warm acclimation while Cu and hypoxia clearly increased transcript levels of genes encoding for proteins involved in metals detoxification/stress response. Overall, the studies I carried out not only provided the mechanistic underpinnings of responses of fish to thermal stress, hypoxia and Cu, but unveiled novel interactive effects of multiple stressors. Importantly, I showed that mitochondria are a viable platform for integrating effects of multiple stressors.

PREFACE

List of publications arising during candidature

Publications that made part of this thesis

1. **Sappal R**, MacDonald N, Fast M, Stevens D, Kibenge F, Siah A and Kamunde C. 2014a. Interactions of copper and thermal stress on mitochondrial bioenergetics in rainbow trout, *Oncorhynchus mykiss*. Aquatic Toxicology. 157: 10-20.

2. **Sappal R**, MacDougald M, Stevens D, Fast M and Kamunde C. 2014b. Copper alters the effect of temperature on mitochondrial bioenergetics in rainbow trout, *Oncorhynchus mykiss*. Archives of Environmental Contamination and Toxicology. 66: 430-440.

3. **Sappal R**, MacDougald M, Fast M, Stevens D, Kibenge F, Siah A and Kamunde C. 2015a. Alterations in mitochondrial electron transport system activity in response to warm acclimation, hypoxia-reoxygenation and copper in rainbow trout, *Oncorhynchus mykiss*. Aquatic Toxicology. 165: 51-63.

4. **Sappal R**, Fast M, Stevens D, Kibenge F, Siah A and Kamunde C. 2015b. Effects of copper, hypoxia and acute temperature shifts following warm acclimation on mitochondrial oxidation in rainbow trout (*Oncorhynchus mykiss*). Submitted for peer review in Aquatic Toxicology: MS# AQTOX-D-15-00327.

5. **Sappal R**, Fast M, Purcell S, MacDonald N, Stevens D, Kibenge F, Siah A and Kamunde C. 2015c. Transcriptional and functional impacts of multiple stressors on energy metabolism and

stress response in rainbow trout (*Oncorhynchus mykiss*). Submitted for peer review in Environmental Pollution: MS# ENVPOL-D-15-01303R1.

6. **Sappal R**, Fast M, Stevens D and Kamunde C. 2015d. Mitochondrial bioenergetics: a platform to integrate effects of multiple stressors in fish. Review article in preparation.

Publication not included in this thesis

7. **Sappal R**, Fast M, Stevens D and Kamunde C. 2015e. Dependence of mitochondrial dysfunction on copper exposure and reoxygenation duration in rainbow trout, *Oncorhynchus mykiss*. Manuscript in preparation.

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ਨ ਡਰੋਂ ਅਰਿ ਸੋ ਜਬ ਜਾਇ ਲਰੋਂ ਨਿਸਚੈ ਕਰਿ ਅਪੁਨੀ ਜੀਤ ਕਰੋਂ ॥

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LIST OF ABBREVIATIONS

AMPKα1	AMP-activated protein kinase α1
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocase
ATOX1	Antioxidant protein 1
ATP	Adenosine triphosphate
ATP7A	Copper transporting ATPase, alpha polypeptide
ATP7B	Copper transporting ATPase, beta polypeptide
BSA	Bovine serum albumin
CI	Complex I (NADH: ubiquinone oxidoreductase)
CII	Complex II (succinate: ubiquinone oxidoreductase/ succinate dehydrogenase)
CIII	Complex III (ubiquinol: cytochrome c oxidoreductase/cytochrome bc_1 complex
CIV	Complex IV (cytochrome c oxidase/COX)
CV	Complex V (F_1F_0ATP synthase)
cDNA	Complimentary DNA
CNRQ	Calibrated normalized relative quantity
CCS	Cu chaperone for superoxide dismutase
Cu	Copper
Cu^{2+}	Cupric ion
Cu^{+}	Cuprous ion
COX4-1	Cytochrome c oxidase subunit 4 isoform 1

COX4-2	Cytochrome c oxidase subunit 4 isoform 2
Cox17	Cytochrome c oxidase Cu chaperone
Ctr1	Copper transporter 1
CsA	Cyclosporine A
cyt c	Cytochrome c
DCPIP	2,6-dichlorophenolindophenol
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E_a	Activation energy
ED_{50}	Median effective dose
EDTA	Ethylene diamine tetraacetic acid
EF-1α	Eukaryotic elongation factor 1 alpha paralog B
EGTA	Ethylene glycol tetraacetic acid
eIF3	Eukaryotic translation initiation factor 3 subunit 6
ETS	Electron transport system
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
FCCP	Carbonyl cyanide-4-trifluoromethoxyphenylhydrazone
Fe-S	Iron sulfur centre
FMN	Flavin mononucleotide
GRE	Glucocorticoid response element
HSP	Heat shock protein
HSI	Hepatosomatic index

HSD	Honest significance difference
HRO	Hypoxia-reoxygenation
HIF-1α	Hypoxia inducible factor-1α
HVA	Homeoviscous adaptation
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IPCC	Intergovernmental Panel on Climate Change
LON	ATP-dependent protease La
LSD	Least significant difference
MANOVA	Multivariate analysis of variance
mtDNA	Mitochondrial DNA
MIB	Mitochondrial isolation buffer
MRB	Mitochondrial respiration buffer
MTs	Metallothioneins
MT-A	Metallothionein A
MT-B	Metallothionein B
MMP	Mitochondrial membrane potential
MPTP	Mitochondrial permeability transition pore
MRE	Metal response element
MTF-1	Metal-regulatory transcription factor 1
NAC	N-acetyl cysteine
NADH	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA

NTC	No template control
OCLTT	Oxygen and capacity limitation of thermal tolerance
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
P/O	Phosphate:oxygen ratio
pmf	Proton motive force
Q	Coenzyme Q
Q_{10}	Temperature coefficient
RCR	Respiratory control ratio
Rh-123	Rhodamine 123
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPS20	Structural ribosomal protein S20
RQI	RNA quality indicator
RT	Reverse transcription
state 4_{ol}	State 4 plus oligomycin (proton leak)
TCA	Tricarboxylic acid cycle
TMPD	Tetramethyl-p-phenylenediamine
UCPs	Uncoupling proteins
USEPA	United States Environmental Protection Agency
WQC	Water quality criteria

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CHAPTER 1: GENERAL INTRODUCTION

A version of this Chapter is included in a review article:

Sappal R, Fast M, Stevens D and Kamunde C. 2015d. Mitochondrial bioenergetics: a platform to integrate effects of multiple stressors in fish (manuscript in preparation).

1.1 Overview

Many aquatic systems experience changes in their chemical and physical characteristics due both to natural phenomena and consequences of anthropogenic activities. While virtually all characteristics of the aquatic environment are amenable to change, fluctuations in temperature, oxygen (O_2) levels, pH, salinity and chemical constituents are most prevalent. As a corollary, fish in their natural habitats commonly encounter numerous potentially stressful environmental changes either simultaneously or sequentially and the effects of their interactions evoke complex responses that are largely unknown because most studies have evaluated effects and/or mechanisms of action of single stressors at a time. Coping with environmental stress involves behavioural, physiological, biochemical and transcriptional adjustments that require energy expenditure. It can therefore be anticipated that the "powerhouses" of the cell –mitochondria– that generate the majority (>90%) of cellular energy (ATP) would play a key role in response to environmental change. Conversely, the physiological status of the organism can modulate mitochondrial function and this has sparked the notion that mitochondrial physiology may integrate the effects of environmental stressors in fish.

The overarching goal of my thesis was to elucidate the interactive responses of multiple stressors and their underlying mechanisms in fish. Environmental perturbations may disrupt cellular homeostasis by affecting systems that are involved in production, conversion and conservation of energy, ultimately reducing aerobic scope and stress tolerance. In this thesis, I tested the hypothesis that fish experience energy dyshomeostasis during environmental stress and that mitochondrial metabolism can be used as a sensitive endpoint to detect and integrate effects of multiple stressors. While the number of potential stressors is vast I focussed on mitochondrial responses imposed by individual and joint exposures to temperature stress, hypoxia and metals (copper, Cu) because

effects of these stressors are among the most consequential in aquatic systems. Experiments were carried out both *in vitro* and *in vivo* at varying temperatures, O_2 and Cu regimes using rainbow trout, *Oncorhynchus mykiss* and isolated mitochondria with assessment of effects and mechanisms operating at multiple levels of biological organisation.

1.2 Energy homeostasis

Living organisms need a constant supply of energy to fuel activities that are critical for their maintenance and biological functions including growth, reproduction and stress management. The maintenance of stable internal energy stores in the face of changes in energy input and expenditure is known as energy homeostasis. Maintaining energy homeostasis involves the modulation of complex and interwoven pathways that sense energetic status and drive energy production, consumption and storage at the level of the cell and organism.

1.2.1. Organismal energy homeostasis

Energy for carrying out organismal functions is derived from catabolism of complex organic molecules including carbohydrates, proteins and fats present in foods. The maintenance of energy homeostasis at the organism level in vertebrates involves several organ systems that assimilate, breakdown and distribute nutrients and ultimately use them to produce energy (ATP). The entire process of energy production is divided into 3 main stages (Lodish et al. 2000): Stage 1 includes digestion that occurs outside of animal cells and involves enzymatic breakdown of food molecules, Stage 2 starts in the cytosol when nutrients are assimilated by tissues and it ends in the mitochondrion, and Stage 3 occurs solely in the mitochondrion.

During stage 1, the complex energy-bearing organic molecules are broken down into simpler molecules (glucose, amino acids, fatty acids, glycerol and nucleotides) by digestive enzymes and secretions of gastrointestinal tract (from salivary glands, stomach, pancreas, liver, bile) and are then absorbed via the alimentary canal mucosa into blood and transported to the liver via the hepatic portal vein. In the liver these molecules are metabolized and the products (glucose, amino acids and fatty acids) either distributed to other tissues via systemic circulation or stored (as glycogen or fat). Fatty acids also enter the lymphatic system to reach systemic circulation for storage in adipose tissue. Within the various tissues these molecules are used in energy production for growth, repair or maintenance and to support other biological functions.

Several organs including the brain, liver, muscle, pancreas, stomach and intestine regulate energy homeostasis and are in constant communication to maintain a stable metabolic state. Liver metabolism provides fuel/energy source to brain, muscle and other peripheral organs. The liver also produces glucose from glycogen or from lactate, alanine, and glycerol (through gluconeogenesis). The brain is a major consumer of energy produced from carbohydrate metabolism because it has minimal fuel stores and therefore requires a constant supply of energy in the form of glucose. Because fatty acids are bound to albumin they cannot cross the blood brain barrier to act as an energy source for the brain but they are a major fuel source for muscle despite the fact that most of the glycogen is stored in this tissue and can be more readily broken down to glucose as a quick energy source. Kidneys also require large amounts of energy to support their secretory, reabsorptive and excretory functions. Excess glucose is stored in the liver, muscle and kidney as glycogen whereas fatty acids are stored as triglycerides in adipose tissue and muscle for future energy requirement. In times of need, glycogen and triglycerides are broken down to glucose

and fatty acids, respectively, and transported to cells to enter glycolysis or β-oxidation for energy production (Lodish et al. 2000; Nelson and Cox 2008).

1.2.2 Cellular energy homeostasis

Cellular energy is mainly in the form of high energy phosphate bonds of adenosine triphosphate (ATP), also known as the energy currency of the cell. When fuel molecules e.g., glucose and fatty acids reach cells, they enter a variety of metabolic pathways including glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS) and β-oxidation to produce a common end product, ATP. Cellular energy homeostasis is the equilibrium maintained between ATP production and consumption within the cell for normal growth and function. In eukaryotes, >90% of the cellular ATP production occurs in the mitochondria through OXPHOS and only a fraction is synthesized via substrate-level phosphorylation (e.g., glycolysis).

Stage 2 of energy production (Lodish et al. 2000; Nelson and Cox 2008) starts with glycolysis in the cytosol and ends in the mitochondria with production of acetyl-CoA which is further used in the TCA cycle to produce high energy electrons in the form of NADH and $FADH_2$ that are required for OXPHOS. During glycolysis, glucose is converted to pyruvate which is transported into mitochondria and converted (by pyruvate dehydrogenase) to acetyl-CoA. Glycolysis occurs virtually in all tissues and can operate under both aerobic and anaerobic conditions. On the other hand, fatty acids are metabolized through β-oxidation to acetyl-CoA, NADH and $FADH_2$ in the mitochondria with the former entering the TCA cycle and the latter two donating electrons to the electron transport system (ETS) for OXPHOS.

Stage 3 of energy production occurs in the mitochondria. Here, acetyl-CoA produced during glycolysis, β-oxidation and amino acid metabolism is oxidized in the TCA cycle within the

mitochondrial matrix to generate high-energy electron carriers, NADH and $FADH_2$. The high-energy electrons in these carriers are subsequently donated to the ETS and used in a series of redox reactions to produce ATP through OXPHOS (discussed below).

1.3 Mitochondria: history, structure and function

In 1890, Richard Altmann first recognized these organelles and named them "bioblasts" (Ernster and Schatz 1981). Altmann described these organelles as "elementary organisms" living inside the cell and performing vital functions. The current name –mitochondrion– was introduced in 1898 by Carl Benda and originates from two Greek words, "mitos" (thread) and "chondros" (granule). Mitochondria share evolutionary past with prokaryotes and are thought to have developed from proteobacteria/rickettsias. According to the endosymbiotic theory, an aerobic proteobacterium managed to invade and live inside eukaryotes as an endosymbiont. The host cell benefitted from aerobic proteobacterium's ability to extract O_2 to produce energy and hence was able to thrive in O_2-rich environments while all other eukaryotes become extinct. This proteobacterium in return benefitted from a protected stable environment to live in without getting digested and with years of evolution became a mitochondrion (Gray et al. 1999).

Morphologically, mitochondria are double membrane cytoplasmic organelles ranging in size from 1-2 μm (length) x 0.1 to 0.5 (diameter) μm (Palade 1952; Lodish et al. 2000). Their shape and number vary depending upon the organism, tissue, cell type and metabolic energy demand. Imaging techniques have shown that mitochondria are in constant motion and can change their size and shape. Mitochondrial dynamics (shape, size, number, distribution, motility in the cytosol and inheritance during cell division) is controlled primarily by the balance between two processes, mitochondrial fission and fusion (Mishra and Chan 2014). This phenomenon is important not only

for maintenance of mitochondrion integrity but also for its interaction with cytoskeleton and other cell organelles. For example, mitochondria and the endoplasmic reticulum (ER) are joined at multiple contact sites to form interorganellar connections termed as mitochondria-ER associated membranes (MAMs), where the functions of the two organelles ranging from mitochondrial fission, phospholipid biosynthesis to transmission of calcium signals are coordinated (Nicholls and Ferguson 2013).

Mitochondria possess their own DNA (mtDNA: 16,569 bp in humans) which is circular (humans and other vertebrates) and maternally inherited. Mitochondrial proteins are encoded by both mtDNA and nuclear DNA (nDNA), with import of the latter into the organelle occurring via complex posttranslational and co-translational mechanisms (Lecuyer et al. 2007; Ahmed and Fisher 2009; Michaud et al. 2014). The outer mitochondria membrane (OMM) contains large aqueous channels called porins which makes it permeable to ions and molecules of up to 10 kDa (Lodish et al. 2000). The inner mitochondrial membrane (IMM) is envaginated to form projections/folds into the matrix known as cristae that increase organellar surface area and house the ETS. The IMM is impermeable and specific protein transporters are required for translocation of substances in and out of the matrix. The space between the outer and inner membrane is called intermembrane space (IMS) and its contents are similar to cytoplasm because the porins present in OMM allow free movement of ions and small molecules. The IMM plays a major role in OXPHOS by setting the structural barrier for the proton gradient required for ATP synthesis. Because of impermeability of the IMM, the composition of the matrix is different from that of cytoplasm and IMS. The matrix contains metabolites of TCA cycle, β-oxidation and OXPHOS, enzymes for TCA cycle and β-oxidation, mitochondrial ribosomes, rRNA, tRNA and mtDNA. The generic structure of the mitochondrion is shown in Fig. 1.1.

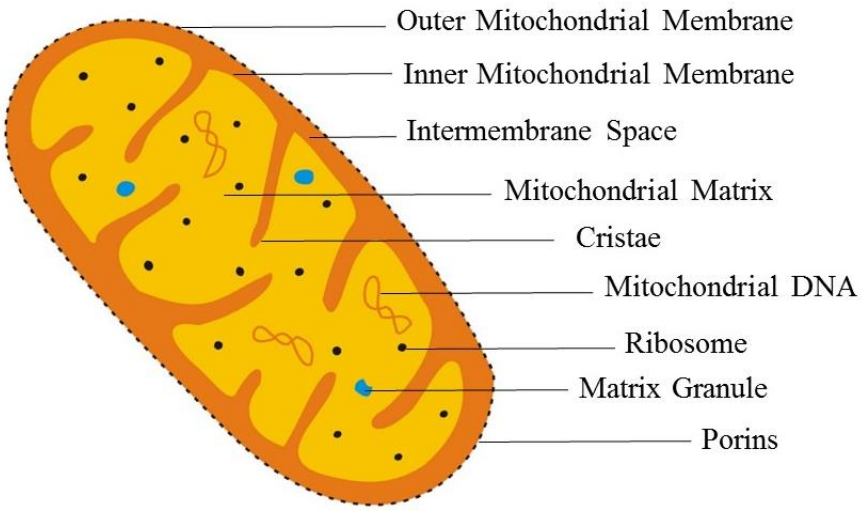

Figure 1.1. The mitochondrion (modified from Nelson and Cox, 2008)

In addition to being responsible for synthesizing ATP, mitochondria are involved in a host of other important cellular functions including urea cycle, heme synthesis, calcium and iron homeostasis, steroidogenesis, intracellular signalling, cell growth and differentiation, apoptosis and thermogenesis (Nelson and Cox 2008; Rizutto et al. 2012).

1.3.1 Electron transport system (ETS)

The mitochondria house complex metabolic pathways including highly integrated enzymatic systems such as the ETS. The ETS consists of four multimeric protein complexes (complex I, II, III and IV: CI, CII, CII and CIV) functionally connected through electron carriers, coenzyme Q/ubiquinone (Q) and cytochrome c (cyt c). The ATP synthase constitutes complex V (CV), the final enzyme of OXPHOS but is not involved in electron transport (Fig. 1.2).

Mitochondrial CI (NADH: ubiquinone oxidoreductase), a 980 kDa protein with 46 subunits (bovine), is the first and largest enzyme complex of the ETS (Hirst et al. 2003). It is L-shaped consisting of 14 central and up to 32 accessory subunits (Hirst et al. 2003). This protein complex has 7 mtDNA and 38 nDNA encoded proteins (Hirst et al. 2003). The peripheral or matrix protruding hydrophilic arm contains flavin mononucleotide (FMN) and iron-sulfur (Fe-S) clusters as redox prosthetic groups whereas the membrane arm contains hydrophobic subunits encoded by mtDNA. The Q-binding site is at the junction between the arms. CI provides the entry point for electrons from NADH to Q while simultaneously pumping protons across the IMM (Brandt 2006; Hunte and Brandt 2010). This complex is subdivided into 3 functional modules: N or input module which oxidizes NADH, Q is the electron output module that reduces quinone, and P module that pumps protons across the membrane. Electron transfer from NADH to Q is entirely confined to N and Q modules. Electrons from NADH enter via FMN and are transferred to a chain of eight Fe-S

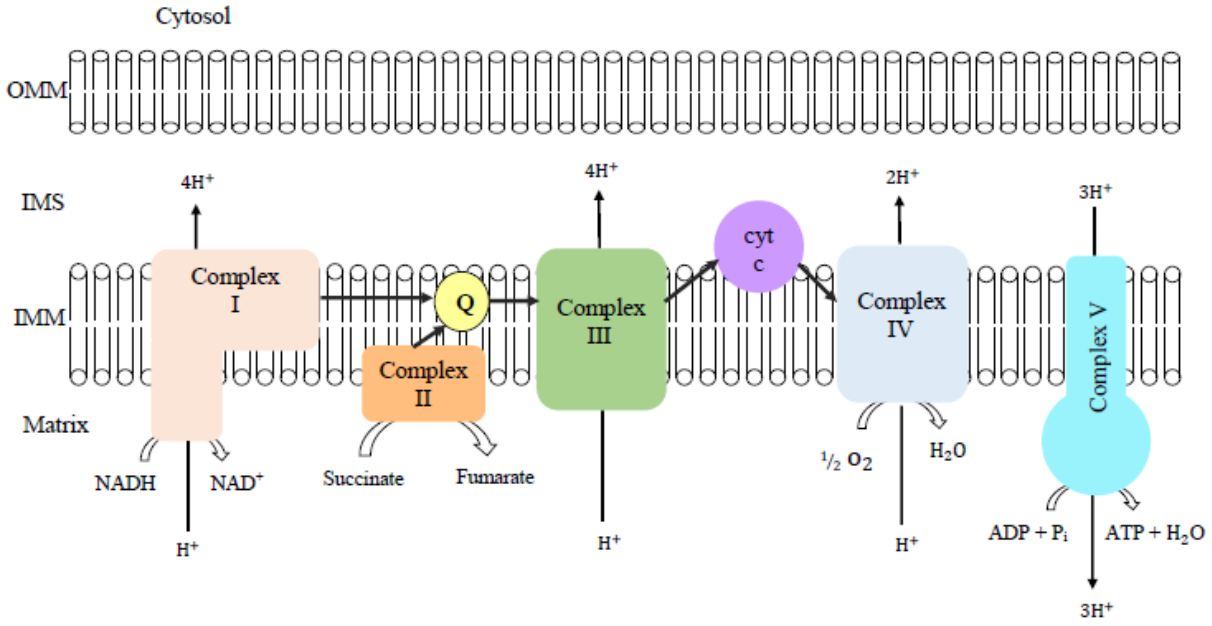

Figure 1.2. The enzyme complexes of mitochondrial electron transport system. Complex I and Complex II of inner mitochondrial membrane (IMM) transfer electrons from reduced nicotinamide adenine dinucleotide (NADH) and succinate, respectively, to coenzyme Q (Q) and then via Complex III and cytochrome c (cyt c) to Complex IV, where they are accepted by oxygen (O_2) to form water (H_2O). This transfer of electrons is coupled with the pumping of protons from the matrix to the inter membrane space (IMS) creating a proton gradient across the IMM that drives adenosine triphosphate (ATP) synthesis at Complex V. Outer mitochondrial membrane (OMM), proton (H^+), adenosine diphosphate (ADP), inorganic phosphate (P_i). Modified from Nelson and Cox (2008).

clusters and finally to Q (Brandt 2006; Nicholls and Ferguson 2013). This electron transfer drives 4 protons from the matrix into the IMS per NADH oxidation.

Complex II (succinate: ubiquinone oxidoreductase/succinate dehydrogenase), a 140 kDa protein, is the only enzyme of the ETS that participates in the TCA cycle and is partially embedded in IMM (Nelson and Cox 2008). It consists of 4 subunits which are entirely encoded by nDNA (Balsa et al. 2012); two of the subunits are hydrophilic (extend into matrix) and the other two are hydrophobic (embedded in IMM). This complex bears 5 prosthetic groups: heme *b*, flavin adenine dinucleotide (FAD) and three Fe-S clusters (Nicholls and Ferguson 2013). It oxidizes succinate to fumarate in a reaction that is coupled with the reduction of FAD to $FADH_2$, which then transfers electrons to the Fe-S clusters and on to Q; heme *b* does not participate in electron transfer (Drose 2013). During this transfer of electrons to Q, no protons are pumped into IMS (Nelson and Cox 2008).

Complex III (ubiquinol: cytochrome c oxidoreductase/cytochrome bc_1 complex), a 250 kDa IMM protein, is a dimer that consists of identical monomers each with 11 subunits of which only one (cytochrome b) is encoded by mtDNA with the other 10 being nDNA encoded (Iwata et al. 1998). The catalytic core of each monomer is made up of 3 subunits, cytochrome b (with its 2 heme groups), Rieske protein with its Fe-S cluster and cytochrome c_1 (Nelson and Cox 2008). Electrons are sequentially transferred from Q to cytochrome b, Fe-S, cytochrome c_1 and finally to cyt c. During this process 4 protons (per electron pair transferred) are pumped from the matrix to IMS. Metabolism of glycerol 3 phosphate (from glycolysis) and β-oxidation of fatty acids also contribute to the electron pool of Q by donating electrons to flavoproteins that later transfer them to Q (Nelson and Cox 2008).

Complex IV (cytochrome c oxidase, COX), a 160 kDa dimeric Cu-containing IMM protein is the terminal complex of the ETS (Nelson and Cox 2008). It consists of 13 polypeptide subunits encoded by nDNA and mtDNA and 4 redox-active metal centres (heme *a*, heme a_3, Cu_A and Cu_B) that participate in electron transfer. The 3 biggest subunits, COX1, 2 and 3 are encoded by the mtDNA and form the functional core of the enzyme complex. Subunits COX1 and 2 perform the electron transfer function whereas COX3 is required for proton pumping, assembly and stability of subunits 1 and 2 (Capaldi 1990; Tsukihara et al. 1995; Barrientos et al. 2002). The remaining 10 subunits and their isoforms are all nDNA encoded and serve regulatory or structural functions. The function and regulation mechanisms of each subunit, especially of those encoded by the nDNA, are largely unknown (Li et al. 2006b). Isoforms of the nDNA encoded subunits modulate the catalytic functions of the mtDNA encoded subunits (Burke and Poyton 1998). One of the main nDNA encoded subunit, COX4, is essential for the proper assembly of COX1, COX2, and COX3 (McEwen et al. 1986; Tsukihara et al. 1996; Nijtmans et al. 1998) and is incorporated during the early stages of COX assembly (Nijtmans et al. 1998). Indeed, this subunit plays a major role in the overall function and regulation of COX enzyme. Redox active Cu_A resides in COX2 and consists of a binuclear centre that accepts electrons from reduced cyt c. The now reduced Cu_A transfers the electrons to heme *a* then to heme *a3*–Cu_B binuclear center, all in COX1. The electrons are finally transferred to O_2 to form water. For each pair of electrons transferred to an oxygen atom, 2 protons are pumped into the IMS (Nelson and Cox 2008).

Complex V or F_1F_0ATP synthase is a 500 kDa protein complex that consists of a hydrophilic F_1 subcomplex with 5 subunits and a hydrophobic membrane embedded F_0 subcomplex with 3 subunits. Only 2 subunits of F_0 subcomplex are encoded by mtDNA. The F_1 catalyzes ATP synthesis while F_0 performs proton translocation. Specifically, when protons from the

intermembrane space diffuse back into the mitochondrial matrix through F_0 subcomplex, a rotary mechanism performed by F_0 and F_1 subcomplexes causes conformational changes at the ATP binding sites enabling ATP synthesis from ADP and P_i. Complex V can also work in reverse direction, acting as a proton pump and performing cellular functions like transmembrane transport of ions or nutrients thus consuming/hydrolysing, instead of synthesizing, ATP (Nelson and Cox 2008).

1.3.2 Oxidative phosphorylation (OXPHOS)

The ETS is the site of OXPHOS, a process that couples oxidation (electron transfer from metabolic reducing equivalents, NADH and $FADH_2$ to O_2 terminally) and phosphorylation (conversion of ADP to ATP) through an energy gradient –the proton motive force (pmf) – across the IMM (Nelson and Cox 2008). For every 3 protons traversing the F_1F_0ATPase complex one molecule of ATP is formed. Briefly, high energy electrons from NADH and $FADH_2$ are transferred to CI and CII, respectively, and are carried by electron carriers (flavoproteins, Fe-S proteins, Q and cytochromes) via CIII to cyt c and subsequently through the redox carriers of the terminal ETS enzyme (COX/CIV) which transfers them to the terminal acceptor, O_2, to form water. Thus O_2 consumption (respiration) is the final step of electron transport and is used as metric of mitochondrial function. During electron transfer, the protons pumped from the matrix to IMS by CI, CIII and CIV generate an electrochemical gradient (pmf). This pmf provides the energy that drives ATP synthesis by CV. In coupled respiration, O_2 consumption is coupled with ATP synthesis, but it is never 100% efficient because some protons re-enter the mitochondrial matrix bypassing CV, and their energy is dissipated as heat instead of being conserved as ATP. This phenomenon is referred to as proton leak.

1.3.2.1 Proton leak

The mechanisms of proton leak have been described in two excellent reviews (Jastroch et al. 2010; Divakaruni and Brand 2011). According to these reviews, the total proton leak is the sum of basal leak and inducible leak (Fig. 1.3). Basal leak is constitutive, unregulated and is mediated by mitochondrial anion carrier proteins, adenine nucleotide translocase (ANT) and the lipid bilayer. It is characteristic of all mitochondria and contributes significantly to basal metabolic rate. ANT-mediated basal leak is found in tissues with high OXPHOS rates and, via mild uncoupling, provides protection against ROS production without major impact on ATP synthesis. Inducible proton leak is regulated by ANT and uncoupling proteins (UCPs) but it can also be activated by fatty acids, superoxide or peroxidation products. Inducible proton leak by UCPs is a characteristic of endotherms and its main role is heat production during non-shivering thermogenesis (Jastroch et al. 2010; Divakaruni and Brand 2011). Thus proton leak has beneficial physiological functions including regulation of metabolic rate, reducing ROS production and thermogenesis, and should therefore not be viewed merely as an inefficiency of the ETS.

1.3.2.2 Electron leak and ROS

In addition, leakage of electrons during OXPHOS results in premature one-electron reduction of O_2 at CI, II and III to form superoxide anion radical (Fig. 1.4), a ROS that is subsequently converted to hydrogen peroxide by superoxide dismutase (Hamanaka and Chandel 2010). It has been estimated that under normal physiological conditions 0.15-2% of the O_2 consumed by the mitochondria is converted to superoxide anion radicals (Hamanaka and Chandel 2010 and references therein); however, with disabled antioxidant system it may reach 11% depending on

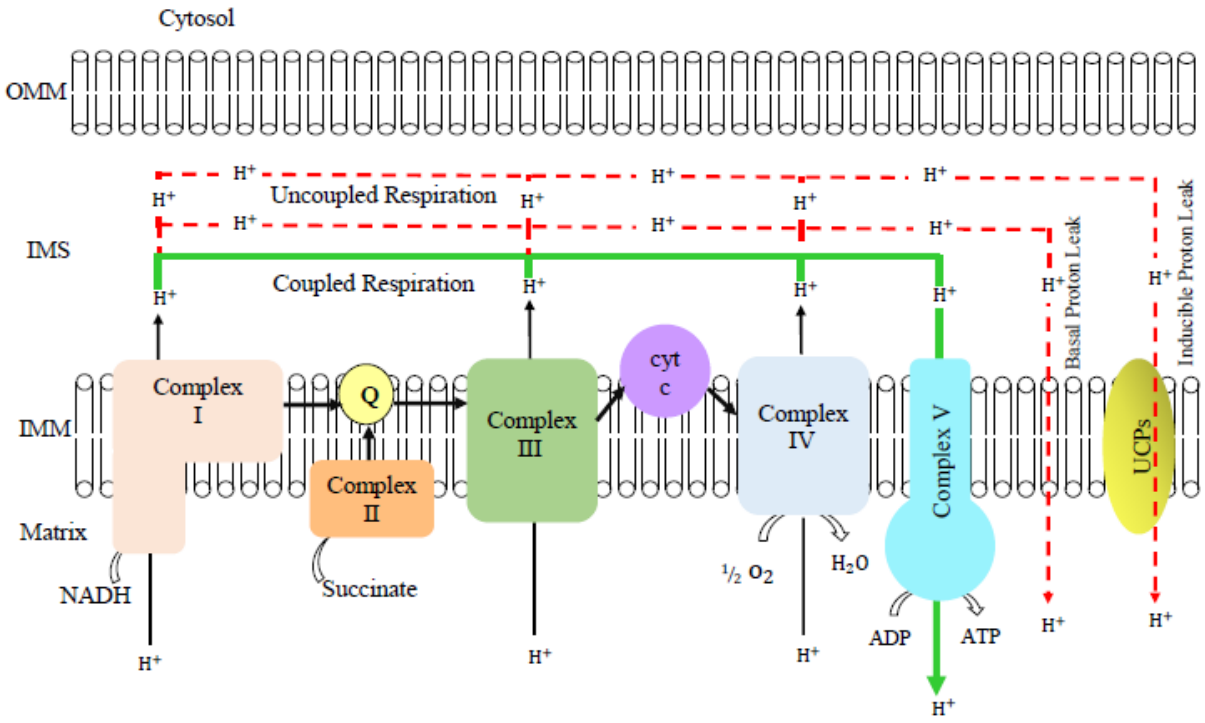

Figure 1.3. Proton pumping and leak across inner mitochondrial membrane (IMM). Outer mitochondrial membrane (OMM), inter membrane space (IMS), nicotinamide adenine dinucleotide (NADH), coenzyme Q (Q), cytochrome c (cyt c), oxygen (O_2), water (H_2O), adenosine triphosphate (ATP), adenosine diphosphate (ADP), proton (H^+), uncoupling proteins (UCPs). Modified from Jastroch et al. (2010) and Divakaruni and Brand (2011).

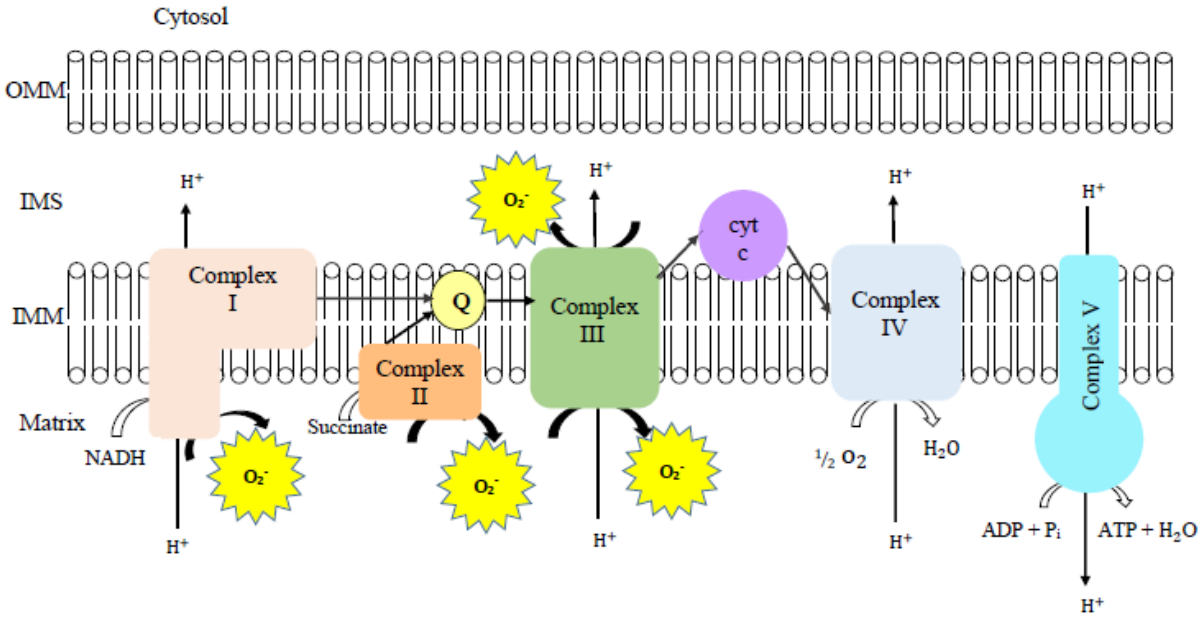

Figure 1.4. Main sites of electron leak and ROS production in the electron transport system. Complex I, II and III produce superoxide anion radicals (O_2^-) in the matrix through incomplete reduction of oxygen (O_2). Complex III also produces superoxides in the inter membrane space (IMS). Inner mitochondrial membrane (IMM), outer mitochondrial membrane (OMM), nicotinamide adenine dinucleotide (NADH), coenzyme Q (Q), cytochrome c (cyt c), water (H_2O), adenosine triphosphate (ATP), adenosine diphosphate (ADP), inorganic phosphate (P_i), proton (H^+). Modified from Hamanaka and Chandel (2010).

the species and respiration rates (Zorov et al. 2014). Moreover, while mitochondrial ROS have historically been viewed as harmful products of the ETS, their role in cellular signaling has recently been recognized (Zorov et al. 2014).

1.3.3 Modulation of ETS respiratory activity and ROS production

The pathways of electron transfer through the ETS and the function of the associated protein complexes were initially discovered using antimetabolites or compounds that modulate specific steps in the process. These compounds have been categorized as a) inhibitors of ETS, b) inhibitors of ANT and c) uncouplers of OXPHOS. Because these compounds alter electron transport and pmf, they ultimately inhibit ATP synthesis. A list of these ETS modulators and their mechanisms of action is provided in Table 1.1 while the inhibitors and uncouplers I used in my experiments are depicted in Fig. 1.5.

Inhibition of electron flow can lead to stimulation or suppression of ROS production. For example, rotenone binds to and blocks CI quinone site, preventing electron transfer to Q thus irreversibly blocking OXPHOS and increasing ROS production (Radad et al. 2006; Deng et al. 2010). Similarly, succinate oxidation occurs in the dicarboxylate binding site and malonate, a succinate analogue, binds competitively to the dicarboxylate site inhibiting succinate dehydrogenase (CII) and suppressing ROS production (Siebels and Drose 2013). In contrast, CI ROS generation increases during blockade of forward electron flow following inhibition of CI or induction of reverse electron transfer (RET) from CII to CI when succinate is used as substrate. Additionally, antimycin A inhibits CIII at Q_i thereby increasing superoxide generation at Q_o centre and hence directing oxidants away from the matrix antioxidant defense system (Guzy and Schumacker 2006). Thus inhibition of CIII with antimycin A increases ROS production in the

Table 1.1. Inhibitors and uncouplers of electron transport system/oxidative phosphorylation.

MOA: mechanism of action. Modified from Nelson and Cox 2008.

Function	Complex	Compound	Target/MOA
Electron transfer inhibition	Complex I	Rotenone Piericidin A Amytal/Amobarbital Chlorpromazine Guanethidine Mercurials Demerol	Prevent electron transfer from Fe-S centre to Q and prevent use of NADH as substrate
	Complex II	Malonate Thenoyltrifluroacetone Carboxin	Prevent electron transfer from Fe-S centre to Q and prevent use of FADH as substrate
	Complex III	Antimycin A British Anti Lewisite Naphthoquinone Hypoglycemic agents Myxothiazol	Block electron transfer from cytochrome b to cytochrome c1 Prevent electron transfer from Fe-S centre to Q
	Complex IV	Cyanide Carbon monoxide Azide Hydrogen sulphide	Inhibit electron flow in cytochrome oxidase
Inhibition of ATP synthase	Complex V	Oligomycin Venturicidin Dicyclohexyl carbodi-imide Aurovertin	Prevent influx of protons through ATP synthase
Inhibition of ATP-ADP exchange Uncoupling of phosphorylation from electron transfer		Atractyloside Bongregate FCCP DNP Valinomycin Thermogenin	Inhibit ANT Hydrophobic proton carriers K^+ ionophore

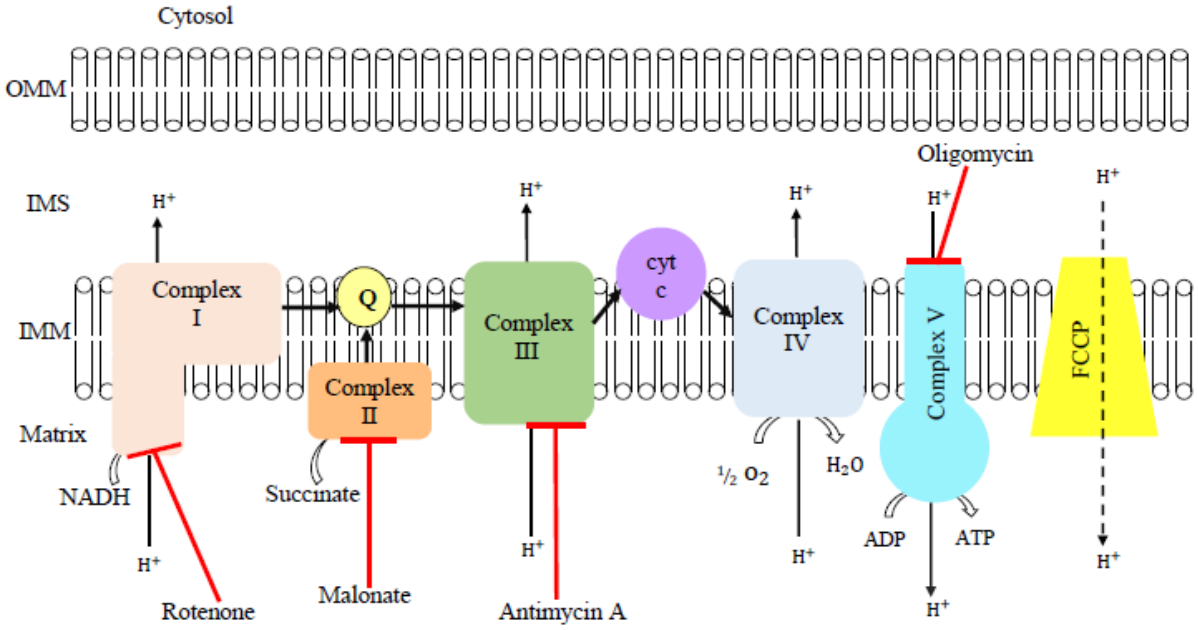

Figure 1.5. Inhibitors and uncouplers of electron transport system used in my experiments (Chapter 2-6). Inner mitochondrial membrane (IMM), outer mitochondrial membrane (OMM), inner membrane space (IMS), nicotinamide adenine dinucleotide, reduced (NADH), coenzyme Q (Q), cytochrome c (cyt c), oxygen (O_2), water (H_2O), adenosine triphosphate (ATP), adenosine diphosphate (ADP), Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), proton (H^+).

mitochondria whereas rotenone inhibition prevents antimycin A induced ROS generation in mitochondria oxidising CI substrates (Chen et al. 2003). The CII inhibitor, malonate, also diminishes antimycin A induced ROS in mitochondria oxidising succinate. Thus inhibition of electron flow upstream of CIII decreases ROS generation (Chen et al. 2003).

1.3.4 Modulation of mitochondrial function by environmental stress

Aerobic organisms rely primarily on mitochondria for their metabolic energy requirements related to basal maintenance and metabolic scope (growth, activity, reproduction). Interference with aerobic metabolism/mitochondrial function can have devastating effects on cellular metabolic processes and homeostasis, decreasing metabolic rate, whole organism performance and in extreme conditions death. Several studies have shown that in aquatic organisms, environmental conditions including fluctuations in O_2 levels, temperature, metals, salinity and pH disrupt energy metabolism (Cherkasov et al. 2006a,b; Sokolova and Lannig 2008; Maes et al. 2013; Ivanina et al. 2012; Sokolova et al 2012). These environmental challenges can lead to changes in mitochondrial content, function and efficiency in order to match energetic demands thus allowing an organism to maintain energy homeostasis (Solaini et al. 2010; Guderley 2011). Because mitochondria are central to energy production, changes in their morpho-functional properties such as rates of electron transfer, availability of substrates, activity of ETS complexes, mitochondrial density and membrane structure might indicate response to environmental and physiological constraints associated with altered energetic demands (Benard et al. 2006, 2010; Pichaud et al. 2013).

1.4 Environmental stress

As a result of natural phenomena and human activities, the physical and chemical properties of many types of aquatic systems have been altered. These alterations include but are not limited to

increased temperature due to emission of greenhouse gases, increased occurrence of eutrophication and hypoxia as a result of excessive anthropogenic input of nutrients and organic matter into water bodies, and metals pollution due to increased mining and industrial activities, disposal of waste and agricultural runoff (Roessig et al. 2004; Diaz and Rosenberg 2008; Diaz and Breitburg 2009). These environmental changes present multiple stresses that may impact aquatic organisms simultaneously or sequentially. Environmental stress has been defined as an environmental condition that causes loss of fitness of an organism or a population (Schulte 2014). Although numerous studies have been undertaken to investigate the effects of stressors in aquatic organisms, the majority assessed a single stressor at a time and rarely more than one. Subsequently, our understanding of the effects, including the underlying mechanisms, of multiple stressors in aquatic organisms remains deficient. The following sections will discuss the three important stressors studied here: temperature, hypoxia and Cu in aquatic systems.

1.4.1 Temperature

The biosphere has warmed considerably in recent years and over the last 1400 years, 1983-2012 was the warmest 30-year period in the Northern Hemisphere (IPCC 2014). Temperature data from land and ocean surfaces show a global temperature increase of 0.65-1.06 °C for the period 1880 to 2012 (IPCC 2014). Though solar radiations, volcanic eruptions and natural emission of greenhouse gases also impact temperature variability, they do not fully explain the warming since the mid-20th century (NRC 2010). Since the pre-industrial era, fossil fuel burning and other human activities have increased atmospheric concentrations of the greenhouse gases carbon dioxide, methane and nitrous oxide (IPCC 2014). These greenhouse gases are believed to be the main cause of the warming observed since the mid-20th century because they trap infra-red radiation (heat) in the atmosphere and do not allow heat to escape into space, resulting in warming of the earth. Warming

of oceans is unequivocal because oceans absorb 90% of the energy (heat) from the atmosphere. Globally, sea surface temperatures have increased on average by 0.11 °C from 1971-2010 (IPCC 2014) and current predictions indicate that the next 20 years (2016-2035) will see an increase in global mean surface temperatures of 0.3-0.7 °C, reaching 1.5 °C by the end of the 21st century (IPCC 2014). Importantly, the frequency of temperature extremes (both cold and warm) is projected to increase, with cold regions experiencing fewer cold days and more warm days thus warming at a faster rate. Warming oceans impact both natural and human systems, by increasing water column stratification and reducing mixing of nutrients, changing precipitation patterns, melting snow and ice cover and raising sea levels, altering hydrological systems, affecting quantity and quality of water resources and changing weather patterns. Global warming is a threat to global diversity as it is already affecting terrestrial, freshwater and marine ecosystems. In the aquatic environment, climate change affects growth, survival, fecundity, migration and reproduction of fish (Crozier and Hutchings 2014); it is therefore important to study effects of temperature in these organisms in order to predict and mitigate the effects of global climate change.

1.4.1.1 Effect of temperature on fish physiology

Temperature is considered the master abiotic factor that has profound effects on the biology of all organisms (Fry 1947). For physiological processes and chemical reactions, the effect of temperature can be explained using the temperature coefficient (Q_{10}), the change in reaction rate over temperatures that differ by 10 °C. Typically, biochemical reactions have Q_{10} of 2 indicating that rates double with every 10 °C change in temperature. In reality, however, at biologically relevant temperatures, Q_{10}s of biological processes are between 1 and 3. Conversely, the Q_{10} is 1 if the rate of a reaction is independent of temperature.

As a result of environmental change or biological phenomena, fish commonly encounter sub-optimal temperatures and have to make requisite biological adjustments in order to survive. Because most fish are ectotherms and their body temperatures vary directly with that of the external (water) environment. As a result, temperature has profound effects on fish at all levels of biological organization (Crozier and Hutchings 2014; Schulte 2015). The nature of these effects varies according to the timescale of the temperature change: short term or acute, medium term or acclamatory, and long term or evolutionary changes (Willmer et al. 2005).

Acute changes may involve modulation of enzyme activity (in a few seconds to several hours) through hormones or the nervous system. At the molecular level, acute temperature influences intermolecular bonds/interactions that determine the three-dimensional structure and function of biomolecules including proteins, nucleic acids and biological membranes (Guderley 2011). Both extremes of temperature are deleterious, with very high temperature denaturing and destabilizing proteins whereas very low temperatures make proteins too rigid to function (Guderley 2011). Acute changes also lead to induction of stress proteins known as heat shock proteins (HSP) which play a key role in processing proteins altered by heat stress. The induction or expression of these proteins occurs within minutes to hours. In contrast, acclimatory changes to temperature occur over days to weeks and affect mainly the concentration of enzymes through protein synthesis and degradation. One of the most important acclimatory processes induced by temperature is homeoviscous adaptation (HVA) that maintains the integrity of biological membranes so that membrane-embedded proteins can function properly (Willmer et al. 2005; Guderley 2011; Moyes and Ballantyne 2011). HVA maintains membrane fluidity at new temperatures by modifying lipid composition of cell membranes through balancing the level of saturation and unsaturation of fatty acids. Generally at low temperatures there is an increase in unsaturated fatty acids while at high

temperatures there is an increase in saturated fatty acids (Moyes and Ballantyne 2011). Lastly, evolutionary changes mainly occur at the genetic level and involve changes in protein structure or sequences either through mutations or natural selection with evolution of more suitable enzymes/proteins for particular thermal regimes (Willmer et al. 2005).

1.4.1.2 Effects of temperature on mitochondrial function

Individual components of the complex and highly integrated mitochondrial biochemical machinery are temperature-sensitive (Blier and Lemieux 2001) and temperature changes in fish have been shown to alter mitochondrial function (Hardewig et al. 1999a; Bouchard and Guderley 2003; Iftikar et al. 2015). Highly elevated temperatures impair mitochondrial oxidative metabolism leading to disruption of cellular ATP production and cell death (Hand and Menze, 2008). Specifically, high temperature uncouples mitochondria (Luvisetto et al. 1992; Hilton et al. 2010) thereby decreasing the efficiency of OXPHOS (Sommer et al. 1997; Hardewig et al. 1999a; Portner et al. 1999a) which in turn restricts ATP production. High temperature also induces oxidative stress (Abele et al. 1998, 2002) by accelerating mitochondrial ROS formation (Abele et al. 1998, 2002; Keller et al. 2004; Heise et al. 2006) leading to oxidative cellular damage. Lastly, heat stress can cause loss of IMM barrier properties, with increased permeability and reduced membrane stability (Zukiene et al. 2007, 2010).

In addition to the effects of acute temperature change, mitochondrial metabolism is also a primary target of acclimatory responses during persistent thermal change in fish (Bouchard and Guderley 2003). Thermal acclimation alters mitochondrial structure and function thereby aligning energy production and consumption with the demands of the new thermal regime (Portner et al. 2005). Specific changes imposed by acclimation that compensate for thermal change include modification

of mitochondrial oxidative capacities through changes in IMM phospholipid fatty acid composition and ETS protein levels (Bouchard and Guderley 2003). Concurrent with membrane modifications and protein content changes, thermal acclimation also alters the catalytic capacities of enzymes that mediate aerobic energy production (Thibault et al. 1996; Bouchard and Guderley 2003; Kraffe et al. 2007).

Notably, the nature of the changes imposed by acclimation depends on the direction of temperature shift. Cold acclimation increases mitochondrial density (Johnston et al. 1998; Sommer and Portner 2002) and/or oxidation capacity of the individual mitochondrion, resulting in overall increase in oxidative capacity (Hardewig et al. 1999a) and higher OXPHOS rates (Guderley and Johnston 1996; St-Pierre et al. 1998; Hardewig et al. 1999a). It also increases activities of ETS enzymes (Crockett and Sidell 1990; Guderley and St-Pierre 2002; Lannig et al. 2003) through changes in catalytic capacity and increases in amounts of enzyme protein and mitochondrial membrane fluidity (Seebacher et al. 2010). In contrast, warm acclimation down-regulates mitochondrial aerobic capacity (Guderley and Johnston 1996; Guderley and St-Pierre 2002; Lannig et al. 2005; Seebacher et al. 2010) and decreases OXPHOS efficiency (Bouchard and Guderley 2003). Overall, many studies have explored the role of mitochondria in thermal adaptation/acclimation culminating in the hypothesis that these organelles are critical in determining species' thermal limits and distribution (Somero 2002, 2011; Portner and Knust 2007; Portner and Farrell 2008).

1.4.2 Hypoxia

1.4.2.1 Hypoxia in aquatic ecosystems

Hypoxia, defined as dissolved O_2 (DO) concentrations less than 5-6 (freshwater) or 2-3 (marine and estuarine environment) mg O_2/l (Diaz and Rosenberg, 1995; Kalff 2000) can occur in aquatic

environments naturally (e.g., seasonally) or due to human activities. Atmospheric diffusion and photosynthesis are the main sources of O_2 in aquatic systems (Kalff 2000; Stickney 2000) while aerobic respiration by biota consume and can deplete DO levels in the water column. Environmental hypoxia can be caused by temperature fluctuations, low photosynthetic activity and low flow or stagnation of water (Karim et al. 2003; Diaz and Breitburg 2009; Martinez et al. 2011). As well, nutrient enrichment due to eutrophication increases growth and respiratory activity of phytoplankton while microbial decomposition of dead phytoplankton increases the biological O_2 demand leading to decreased O_2 levels in the water.

1.4.2.2 Effect of hypoxia on physiology of aquatic organisms

Hypoxia is currently considered as one of the most critical problems of world's hydrosphere and has profound detrimental effects in aquatic systems. O_2 is important for all aspects of aerobic organisms including survival, growth, locomotion and reproduction (Chapman and McKenzie 2009). Reduced levels of O_2 can reduce habitat quality and limit species distribution, leading to decline in populations and changes in community structures, ultimately altering species diversity and richness (Dauer 1993; Diaz and Rosenberg 1995). Fish respond to hypoxia through a wide range of physiological, biochemical, molecular and behavioural mechanisms (Diaz and Brietburg 2009; Richards et al. 2009) that are determined by the duration and severity of hypoxia exposure as well as the fish species and habitat. The immediate response to hypoxia is behavioral wherein fish avoid areas of low, in favor of those with sufficient, O_2 levels. However, when hypoxia is prolonged fish strive to sustain O_2 delivery or limit harmful effects of O_2 depletion while conserving energy by improving efficiency of ATP generation and limiting its consumption (Richards et al. 2009).

Hypoxia-sensitive species like rainbow trout are highly prone to harmful effects of O_2 deprivation as compared with hypoxia-tolerant ones. Their hypoxia sensitive cells lack the ability to produce sufficient amounts of ATP or conserve it during hypoxia/anoxia resulting in biochemical events that eventually lead to cell death. In these fish, cells try to compensate for reduced OXPHOS by using internal carbohydrate reserves to generate ATP via anaerobic glycolysis (Randall et al. 1992; Hochachka 1997; West and Boutilier 1998); however, anaerobic ATP production is inefficient and quickly exhausts the fuel reserves. Under conditions of limited fuel and ATP, cells cannot maintain ionic and osmotic equilibrium and undergo death by necrosis.

Some fish species try to maintain O_2 delivery by increasing respiration rates and water flow over the gills, number of RBCs, O_2 binding capacity of hemoglobin, and erythropoietin levels in kidney and spleen (Richards 2009; Wells 2009), measures that collectively increase the O_2 transport capacity of the fish. Fish species that are tolerant to hypoxia are able to conserve energy during hypoxia/anoxia by the phenomenon of metabolic depression wherein they decrease ATP turnover to maintain metabolic energy balance (Krumschnabel et al. 2000; Richards 2009; Speers-Roesch et al. 2010). Indeed, ATP turnover and metabolic rates have been reported to decrease by as much 5-20 times during hypoxia (Storey 1996; Hochachka 1997). Additionally, some species decrease the activity of ATP consuming pathways (protein synthesis, membrane transport/ion pumping activities, gluconeogenesis, urea synthesis, etc), thus reducing cellular respiration rates and O_2 demand (Hochachka et al. 1996; Storey and Storey 2004). Collectively, to survive under hypoxic or anoxic conditions energy balance has to be maintained through effective coupling of energy supply to demand (West and Boutilier 1998); failure results in cellular energy dyshomeostasis with death as the terminal outcome (Boutilier and St-Pierre 2000).

1.4.2.3 Hypoxia and mitochondrial function

Mitochondria are the main consumers of O_2 in cells via OXPHOS, the core metabolic pathway for aerobic ATP production. During hypoxia/anoxia, the final electron acceptor (O_2) is unavailable to quench electrons from COX. This leads to the failure of generation of a proton gradient across the IMM, resulting in accumulation of protons in the mitochondrial matrix and loss of membrane potential. The ensuing lack of pmf to drive ATP synthesis causes the F_1F_0ATPase to start consuming ATP by pumping the protons out of the matrix to re-establish mitochondrial membrane potential. Thus the mitochondrion is essentially transformed from producer to consumer of ATP (Solaini et al. 2010; Galli and Richards 2014). Additionally, when conditions of low O_2 supply persist, inhibition of ATP dependent processes e.g., ion transport (Na^+/K^+ ATPase, Ca^{2+} ATPase) ensues resulting in loss of intracellular ion homeostasis. Notably, this leads to mitochondrial matrix Ca^{2+} overload triggering the opening of mitochondrial permeability transition pore (MPTP), mitochondrial swelling, increased ROS generation and cell death (Santore et al. 2002; Griffiths 2012; Lukyanova 2013). Other hypoxia-induced changes in the mitochondria include alteration in the conformation of ETS CI (Galkin et al. 2009; Murphy 2009), membrane composition (Guzy and Schumacker 2006), mitochondrial mass and oxidative capacity of ETS complexes (Solaini et al. 2010). Because hypoxia also suppresses the expression of some of the mtDNA and nDNA encoded proteins, the resultant reduced protein synthesis contributes to decreased ETS capacity (Vijayasarathy et al. 2003; Heerlein et al. 2005).

1.4.2.4 Hypoxia-reoxygenation (HRO)

Hypoxic episodes *in vivo* and *in vitro* are commonly followed by re-oxygenation (reintroduction of O_2) giving rise to the term hypoxia-reoxygenation (HRO). HRO imposes greater damage than

hypoxia alone because reintroduction of O_2 causes a massive burst of ROS production (Caraceni et al. 1995; Korge et al. 2008) which not only cause oxidative damage but activates signaling cascades that, among other effects, promote Ca^{2+} uptake (Boutilier and St-Pierre 2000; Li and Jackson 2002). These effects lead to the opening of MPTP which in turn increases the permeability of IMM resulting in mitochondrial swelling and collapse of the IMM potential. If the MPTP remains open it can result in release of cytochrome c into the cytosol activating apoptotic cell death pathways (Galli and Richards 2014; Lukyanova 2013). In severe cases MPTP opening is followed by a catastrophic decline in ATP levels resulting in necrotic cell death (Santore et al. 2002; Griffiths 2012; Lukyanova 2013).

1.4.3 Copper

1.4.3.1 Copper in the environment

Copper and its compounds are found naturally in the earth's crust and have been used by man since prehistoric times due to the valuable physicochemical properties of this metal including malleability, ductility, high electrical and thermal conductibility, and low chemical reactivity. Cu is released into the environment through both natural and anthropogenic processes. Natural discharges into air, water and soil occur through volcanic eruptions, forest fires, natural weathering of rocks/minerals, wind-blown dust, decaying vegetation and sea spray (WQC 1987, Pacyna et al. 1995). The combination of these natural sources and anthropogenic inputs from agriculture, municipal effluents, mining, smelting, manufacturing and harboring activities have made Cu a serious pollutant in many aquatic ecosystems worldwide (Castilla and Nealler 1978; Bryan and Langston 1992; Kennish 1997; Correa et al. 1999; Sanchez et al. 2005). Although Cu (as copper sulfate) is used as a pesticide (algaecide, fungicide, and/or mildewcide) in aquaculture and

agriculture, increased Cu levels in aquatic environments originate mainly from industrial activities (Castilla and Nealler 1978; Schlotfeldt 1992; USEPA, 2007). In freshwater systems, the natural background concentration of Cu is usually in the range of 0.2-30 µg/l but anthropogenic sources can increase these levels to 100 µg/l or more in mining areas (USEPA, 2007).

1.4.3.2 Cu in biology

Cu is an essential micronutrient, necessary for vital biological processes in both prokaryotes and eukaryotes (Watanabe et al. 1997; Pena et al. 1999; Uauy et al. 1998; Festa and Thiele 2011). It is required for growth and key cellular functions including electron and O_2 transport (aerobic metabolism), enzyme catalysis and detoxification of ROS (Taylor and Anstiss 1999; Salviati et al. 2002; Grosell 2012). The biological activity of Cu is primarily derived from its ability to cycle between a stable oxidised Cu (II) and an unstable reduced Cu (I) state (Pena et al. 1999). Because of this special redox chemistry Cu is used in cuproenzymes e.g., cytochrome c oxidase, Cu/Zn superoxide dismutase, lysyl oxidase, dopamine-β-hydroxylase, ceruloplasmin, etc., as redox center. Figure 1.6 shows the key cuproproteins and their functions.

Though Cu is essential, it is toxic at high concentrations (Pena et al. 1999; Grosell 2012). Excess and unsequestered Cu promotes production and accumulation of ROS in cells through Haber-Weiss and Fenton reactions (Gaetke and Chow 2003; De Boeck et al. 2004; Gomiero and Viarengo 2014) resulting in oxidative stress (Stohs and Bagchi 1995; Valko et al. 2005). Oxidative stress damages key biomolecules including membrane lipids, nucleic acids and proteins (Gaetke and Chow 2003; Vutukuru et al. 2006; Craig et al. 2007; Grosell 2012) ultimately resulting in cell death (Halliwell and Gutteridge 1984; Aust et al. 1985; Goldstein and Czapski 1986).

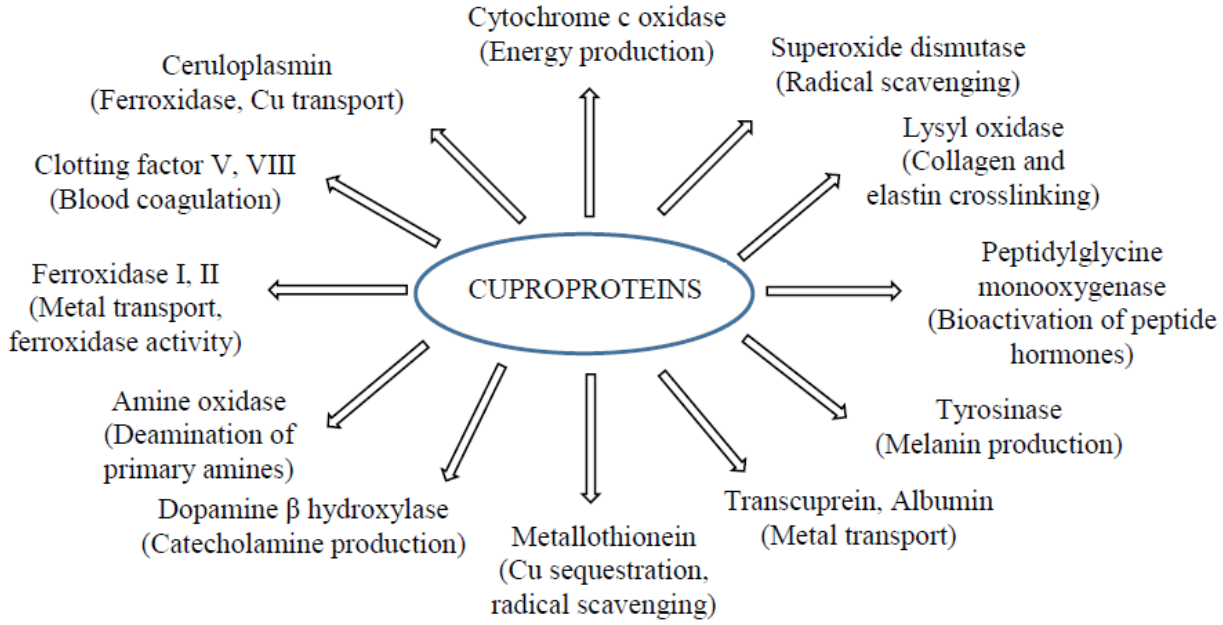

Figure 1.6 Cuproproteins and their functions. Modified from Pena et al. (1999) and Uauy et al. (1998).

Cu also ectopically binds with high affinity to thiol groups of enzymes thereby impairing their function (Letelier et al. 2005), and may displace other essential metals such as zinc from their proteins.

When Cu levels in aquatic systems are elevated, the metal is taken up and accumulated by resident organisms such as fish and may impair growth, development, reproduction and even cause death depending on the exposure concentration (Grosell 2012 and references within). The mechanism underlying these apical effects of Cu include disruption of respiration and cellular oxidative metabolism (De Boeck et al. 1995; Beaumont et al. 2003), oxidative stress (Pourahmad and O'Brien, 2000) and ionoregulatory failure (Grosell et al. 2002; Lushchak 2011; Grosell 2012).

1.4.3.3 Copper homeostasis

Given the essential and deleterious actions of Cu, biological systems require regulatory mechanisms to allow for safe utilization of this metal (Pena et al. 1999; Bury et al. 2003; Kamunde and Wood 2004; Grosell 2012). In fish Cu can be acquired from food via the intestine or from water via gills. In the intestine it is absorbed into blood where it binds to albumin and amino acids for transport to the liver which plays a central role in Cu homeostasis (Pena et al. 1999; Bury et al. 2003; Grosell 2012). Specifically, the liver is important for storing and loading Cu to cuproproteins as well as excreting the excess metal into bile.

Regardless of the cell type and route of uptake, reductase enzymes present on the apical membrane of cells reduce Cu from the cupric (Cu^{2+}) to the cuprous (Cu^{+}) state. The Cu^{+} ions then cross the plasma membrane into cytosol via high-affinity Cu transporter (Ctr1), divalent metal transporter or the epithelial sodium channel (Grosell 2012; Bury et al. 2003). Intracellularly Cu is delivered to three pathways by Cu chaperones: ATOX1 (Antioxidant protein 1) to the secretory pathway,

CCS (Cu chaperone for superoxide dismutase) for metallation of cytoplasmic Cu/Zn-superoxide dismutase (SOD1) and Cox17 (cytochrome c oxidase Cu chaperone) for metallation of COX and mitochondrial SOD1 (Bartnikas and Gitlin 2001; Grosell 2012). ATOX1 delivers Cu to Cu-transporting p-type ATPases (ATP7A and ATP7B), which then deliver it to the trans-Golgi network for incorporation in cuproproteins. Excess Cu causes the Cu-ATPases to relocalize to vesicles in the proximity of either apical (ATP7B) or basolateral (ATP7A) membrane thus allowing Cu efflux/excretion (Lutsenko 2010). Additionally, Cu may be bound by ligands, e.g. glutathione, amino acids and metallothioneins, MTs (located in the cytosol and IMS) that not only play a role in detoxifying excess metal but are involved in intracellular Cu metabolism (Pena et al. 1999; Bury et al. 2002; Bertinato and L'Abbe 2004; Grosell 2012). Overall, Cu homeostasis demands coordination of its uptake, trafficking, storage and excretion via interactions of various transport proteins, chaperones and vesicles.

1.4.3.4 Effect of Cu on mitochondrial function

The mitochondrial matrix contains a bioactive Cu pool that acts as a dynamic rheostat by either expanding or contracting in response to changes in cellular Cu status. Cu is required in the mitochondria for metallation and assembly of COX and SOD1, enzymes that play major roles in aerobic metabolism and protection against oxidative stress, respectively (Leary et al. 2009). Despite its requirement for aerobic metabolism, when in excess Cu has been shown to induce mitochondrial dysfunction by interacting with ETS complexes and inhibiting OXPHOS, collapsing mitochondrial membrane potential, inducing swelling through MPTP opening and increasing ROS production (Krumschnabel et al. 2005; Collins et al. 2010; Belyaeva et al. 2011; Chapter 2 and 3). Some studies also have reported that Cu impairs the activities of glycolytic and TCA cycle enzymes, further compromising mitochondrial function (Lauer et al. 2012). Worthy of

note is that Cu accumulates in the mitochondria of fish tissues following experimental exposure of the metal or from anthropogenically contaminated natural water systems (Couture and Kumar 2003; Kamunde and McPhail 2008).

1.5 Interactions of multiple stressors

Although aquatic organisms normally are exposed to multiple stressors in their natural habitats, our understanding of the interactive responses and their underlying mechanisms is limited (Heugens et al. 2001; Schiedek et al. 2007; Sokolova and Lannig 2008) because most studies have examined the effects of single stressors. Multiple stressors can evoke overlapping cellular responses or affect related cellular pathways leading to additive, synergistic, or antagonistic effects (Folt et al. 1999; Christensen et al. 2006; Altshuler et al. 2011). Importantly, a better understanding of the interactive effects among stressors has implications for accurate prediction of impacts of environmental stressors in aquatic systems.

1.5.1 Interactions of thermal stress, Cu and hypoxia

It is evident from the foregoing sections that temperature fluctuations, hypoxia and metals pollution are among the most important stress factors in present day aquatic systems. Moreover, there is ample evidence suggesting that these factors are linked physically and functionally and therefore can evoke interactive responses in aquatic organisms. For example, according to the concept of oxygen and capacity limitation of thermal tolerance (OCLTT), at warm and cold temperatures, aerobic metabolism would be compromised due to inequality between mitochondrial O_2 requirement and O_2 delivery or by mitochondrial catalytic constraints (Portner 2001, 2010). Based on this concept, high temperature reduces tolerance to hypoxia and hypoxia reduces tolerance to high temperature. Importantly, high temperatures, e.g., due to seasonal changes or

global warming (Diaz and Rosenberg 2008; Roze et al. 2013) in association with hypoxia due to anthropogenic inputs of nutrients and organic matter (Ochumba 1990; Verschuren et al. 2002) are encountered in several freshwater and marine costal ecosystems (Ficke et al. 2007; Rabalais et al. 2009). Because temperature has an inverse relationship with O_2 solubility and a direct relationship with respiration rate/metabolic activity (Guderley 2004; Sorensen et al. 2014), the demand of aquatic biota for O_2 increases with temperature and is exacerbated by the decrease in water O_2 concentration (Diaz and Rosenberg 2008).

Although existing research does not comprehensively address interactions between temperature and pollutants (Sokolova and Lannig 2008), temperature fluctuations influence physiological state of aquatic organisms and alter how they respond to metals exposure (Sokolova 2004; Sokolova and Lannig 2008; Ivanina et al. 2009). It has been shown that temperature modifies the environmental fate and behaviour of metals (Sokolova et al. 2012; Sokolova 2013), influencing accumulation and elimination with implications for toxicity. Specifically, Cu toxicity in aquatic systems is highly influenced by physico-chemical factors (Grosell 2012; Mustafa et al. 2012) via their effects on both Cu bioavailability and physiology of exposed organisms (Flemming and Trevors 1989).

Lastly, of equal importance is that the adaptive responses/mechanisms that fish utilize to handle hypoxia or heat stress are compromised by Cu while temperature and/or hypoxia impair the ability of fish to combat Cu toxicity (De Boeck et al. 1995; Sampaio et al. 2008; Lapointe et al. 2011). Hypoxia and temperature stress could also alter Cu toxicity by increasing metal absorption secondary to increased membrane permeability and elevated gill ventilation and respiratory stroke volume (Fernandes et al. 1995). Interestingly, interactive responses have been observed at the genome level wherein combined exposure to high temperature and Cu enhanced transcription of

genes encoding for proteins involved in energy metabolism and metals detoxification (Lapointe et al. 2011).

1.5.2 Mitochondria as a target for multiple stressors

While significant strides have been made toward understanding the interactions of multiple stressors, the bioenergetic aspects of temperature-hypoxia-metals interactions, have not been sufficiently studied in fish. Mitochondrial energy metabolism has been used to study the interactive effects of several stressors including temperature, metals, hypoxia, pH and salinity on marine ectotherms especially oysters (Cherkasov et al. 2006a,b; Sokolova and Lannig 2008; Sokolova et al. 2012; Sokolova 2013). These studies have shown that exposure to combined temperature and cadmium stress evokes synergistic responses on mitochondrial aerobic capacity. Furthermore, elevated temperature increases the sensitivity of mitochondria to metals and in turn metals impair the ability of mitochondrial enzymes to respond to changes in environmental temperature (Sokolova 2004; Lannig et al. 2005; Ivanina et al. 2008; Sokolova and Lannig 2008; Lapointe et al. 2011). While recent studies using isolated fish mitochondria have reported that hypoxia (with reoxygenation) and temperature sensitize mitochondria to Cd (Onukwufor et al. 2014, 2015) and Cu (Chapters 2-4), fish based studies are generally of limited number and diversity.

1.6 Hypothesis and specific objectives

It is apparent that three commonly encountered aquatic systems stressors –temperature, hypoxia and Cu– individually impair fish physiology but studies on their joint responses on energy metabolism are in a nascent state. My research therefore tested the central hypothesis that the costs and consequences of mounting a response to one stressor compromise the ability of fish to handle an added stressor. Because the effects of temperature, hypoxia and Cu appear to converge on

mitochondria I explored the utility of energy metabolism in delineating the individual responses and integrating the joint effects of these stressors. I predicted that hypoxia and temperature would sensitize fish to Cu by altering mitochondrial function and impairing the ability of fish to effectively mount a stress response. I adopted a holistic approach wherein organismal, tissue, biochemical and molecular endpoints were measured *in vitro* and *in vivo* to gain insights into the mechanisms and significance of the interactive effects of temperature, hypoxia and Cu stress on energy homeostasis.

The specific objectives were:

1) To elucidate the individual and interactive effects of Cu, hypoxia and temperature on mitochondrial bioenergetics.

2) To probe the influence of warm temperature acclimation on mitochondrial ability to handle acute temperature shift, hypoxia-reoxygenation and Cu stress.

3) To determine how warm acclimation, hypoxia and/or Cu exposure on fish modulates mitochondrial transcriptional and biochemical-functional responses.
4) To unveil the independent and joint actions of warm acclimation, hypoxia and Cu on energy sensing and metal handling/stress response.

CHAPTER 2

INTERACTIONS OF COPPER AND THERMAL STRESS ON MITOCHONDRIAL BIOENERGETICS IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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2.1. Abstract

Thermal stress may influence how organisms respond to concurrent or subsequent chemical, physical and biotic stressors. To unveil the potential mechanisms via which thermal stress modulates metals-induced bioenergetic disturbances, the interacting effects of temperature and Cu were investigated *in vitro.* Mitochondria isolated from rainbow trout livers were exposed to a range of Cu doses at three temperatures (5, 15 and 25 ^{o}C) with measurement of mitochondrial CI-driven respiratory flux indices and uncoupler-stimulated respiration. Additional studies assessed effects of temperature and Cu on CI enzyme activity, induction of MPTP, swelling kinetics and MMP. Maximal and basal respiration rates, as well as the proton leak, increased with temperature with the Q_{10} effects being higher at lower temperatures. The effect of Cu depended on the mitochondrial functional state in that the maximal respiration was monotonically inhibited by Cu exposure while low and high Cu concentrations stimulated and inhibited the basal respiration/proton leak, respectively. Importantly, temperature exacerbated the effects of Cu by lowering the dose of the metal required for toxicity and causing loss of thermal dependence of mitochondrial respiration. Mitochondrial CI activity was inhibited by Cu but was not affected by incubation temperature. Compared with the Ca positive control, Cu-imposed mitochondrial swelling exhibited variable kinetics depending on the inducing conditions, and was highly temperature-sensitive. A partial reversal of the Cu-induced swelling by cyclosporine A (CsA) was observed suggesting that it is in part mediated by MPTP. Interestingly, the combination of high Cu and high temperature not only completely inhibited mitochondrial swelling but also greatly increased the RCR relative to the controls. Copper exposure also caused marked MMP dissipation which was reversed by N-acetyl cysteine and vitamin E suggesting a role of ROS in this response. Taken together, Cu impairs OXPHOS in part by inhibiting the ETS, stimulating proton leak, inducing MPTP and dissipating

MMP, with high temperature exacerbating these effects. Thus environmental temperature rise due to natural phenomenon or global climate change may sensitize fish to Cu toxicity by exacerbating mitochondrial dysfunction.

2.2. Introduction

Temperature is considered the abiotic master factor (Brett 1971) and arguably is the most dominant modulator of biological processes at all levels of organization (Hochachka and Somero 2002; Roessig et al. 2004; Schulte et al. 2011; Sunday et al. 2012). While fluctuating environmental temperatures undoubtedly present a challenge to all species, they are of the greatest concern in ectothermic organisms such as fish whose body temperatures conform with those of their environment (Hochachka and Somero 2002; Guderley and St-Pierre 2002). It is predicted that the frequency and magnitude of temperature fluctuations, as well as the mean temperatures, in aquatic systems will increase due to anthropogenically-imposed global climate change (Fickie et al. 2007; Trenberth et al. 2007; Doney et al. 2012), thus imposing greater stress on aquatic ectotherms. To cope with temperature change, aquatic ectotherms utilize a wide array of molecular, biochemical, physiological and behavioral mechanisms (Hochachka and Somero, 2002; Schulte et al. 2011; Blier et al. 2014). Because the realization of these mechanisms requires energy expenditure, mitochondria, which generate the bulk of cellular energy (ATP), have emerged as fundamental drivers of the response to temperature change (Portner 2002, 2010; Iftikar and Hickey 2013; Blier et al. 2014). Although there have been several studies into effects of temperature on fish physiology, the effect of thermal stress on many aspects of mitochondrial function are still not fully understood (Guderley 2011; Blier et al. 2014).

Temperature fluctuations aside, aquatic systems are also threatened by a wide array of other abiotic and biotic stress factors, typically on local scale. It is therefore important to understand how local stressors affect biological processes in the presence of thermal change. In aquatic systems, metals including Cu constitute an important local stressor because they are commonly encountered at elevated concentrations in aquatic systems due to both natural processes and anthropogenic

activities, and fish are generally very sensitive to them. Therefore a better understanding of the mechanisms of how aquatic organisms respond to metals stress in the context of global climate change (Fickie et al. 2007; Doney et al. 2012) is important to more accurately forecast and monitor the environmental impacts of metals.

Copper is considered a priority pollutant under the USEPA Clean Water Act (http://water.epa.gov/scitech/methods/cwa/pollutants.cfm) indicating that its discharge into aquatic systems is monitored and regulated to minimize adverse effects on aquatic biota. In and of itself Cu is an essential trace element that, under normal conditions, is found at low concentrations in animal cells (Turnlund 1999; Gaetke and Chow 2003) where it serves as a cofactor for catalytic and structural properties of several metalloenzymes involved in critical biological processes including growth, development, and maintenance in all organisms (Turnlund 1999; Peña et al. 1999; Gaetke and Chow 2003). Importantly, Cu is vital for aerobic metabolism because it is a catalytic cofactor of COX, a key mitochondrial ETS enzyme. However, at abnormally high concentrations, Cu evokes numerous adverse reactions leading to disturbance in cellular homeostasis and cell death (Pena et al. 1999; Kim et al. 2008). In freshwater fish, although acute toxicity of Cu is attributed to ionoregulatory failure primarily due to direct effects on gill epithelial cells (Grosell 2012), the intracellular targets for Cu have not been unequivocally identified. Nonetheless, it is believed that a fundamental mechanism of Cu-induced toxicity results from its ability to cycle between Cu^{+} and Cu^{2+} redox states (Stohs and Bagchi 1995; Linder and Hazegh-Azam 1996) thereby generating ROS that subsequently cause damage to cellular constituents (Halliwell and Gutteridge, 1984; Harris and Gitlin 1996; Gaetke and Chow 2005). Because the mitochondria are the primary sites of ROS generation in cells (Chen et al. 2003; Kowaltowski et al. 2009; Murphy 2009), it is feasible that Cu can increase mitochondrial ROS generation and

exacerbate damage to these organelles. Indeed, several studies have shown that mitochondria are sensitive Cu targets in endotherms and ectotherms (Saris and Skulskii 1991; Sokol et al. 1993; Belyaeva et al. 2011; Sappal et al. 2014b, 2015a,b) with key effect being impairment of OXPHOS.

It is well established that the functional integrity of mitochondria is derived primarily from IMM which imparts selective permeability and encloses matrix constituents including the mtDNA and enzymes of the TCA, β-oxidation and OXPHOS. However, mitochondrial functional integrity can be disrupted by several stimuli that cause the opening of a nonspecific mega-channel, the MPTP (Zoratti and Szabò 1995; Crompton 1999; Halestrap et al. 2004; Halestrap 2009). Following MPTP opening, influx of small solutes (<1.5 kDa) and osmotically obliged water into the matrix causes mitochondria to swell, eventually rupturing the OMM and releasing proteins such as cyt c that initiate apoptotic cell death. The MPTP also permits protons to freely flow into the matrix causing collapse of the MMP, disruption of OXPHOS and depletion of ATP culminating in necrotic cell death. Although MPTP has attracted substantial research interest, the mechanisms and kinetics of Cu-induced MPTP and mitochondrial volume changes, including the modulatory effect of temperature on this key mitochondrial phenomenon, have not been well investigated particularly in fish.

In light of the central role of mitochondria in cellular homeostasis and response to stress, as well as the prevailing limited understanding of temperature-metals interactions on energy homeostasis, the present study investigated the effects of Cu and thermal stress on mitochondrial function in rainbow trout. The primary objectives were to elucidate the interactive effects of Cu and temperature on mitochondrial bioenergetics and assess the role of the MPTP and MMP in temperature- and Cu-induced mitochondrial dysfunction. I focused on responses under CI-driven respiratory flux because this enzyme complex singly contributes 40% to the pmf for ATP synthesis

and plays a role in regulating thermal and oxidative stress (Murphy 2009; Efremov et al. 2010; Hilton et al. 2010; Blier et al. 2014).

2.3. Materials and methods

2.3.1. Ethical considerations

All the experimental procedures that fish were subjected to are consistent with the guidelines set out by the Canadian Council on Animal Care as approved by the University of Prince Edward Island Animal Care Committee.

2.3.2. Fish and isolation of hepatic mitochondria

Rainbow trout were purchased from Ocean Trout Farm Inc., Brookvale, PE and maintained in a 250-l tank supplied with flow-through aerated well water (temperature 11°C and pH = 7.5) at the Atlantic Veterinary College Aquatic Facility. The fish were fed daily at 1% body weight with commercial trout chow (Corey Feed Mills, Fredericton, NB) and terminal body weights ranged between 90 and 150 g.

To isolate mitochondria, fish were randomly netted from the holding tank and killed by cephalic blow. Livers were immediately dissected out, rinsed with mitochondrial isolation buffer [MIB: 250 mM sucrose, 10 mM Tris-HCl, 10 mM KH_2PO_4, 0.5 mM EGTA, 1 mg/ml BSA (fatty acid free), 2 µg/ml aprotonin, pH 7.3], blotted dry and weighed. Thereafter mitochondrial isolation was done according to our routine procedure (Adiele et al. 2010; Onukwufor et al. 2014; Sappal et al. 2014b). Briefly, 2 or 3 livers were diced into smaller pieces, pooled and homogenized on ice in 3 volumes of MIB using Potter-Elvehjem homogenizer (Cole Parmer, Anjou, QC) with a loosely fitting Teflon pestle. The crude homogenates were transferred to 2 ml centrifuge tubes and

centrifuged at 800 × *g*, 4 °C for 15 min and the supernatants obtained were centrifuged at 13,000 × *g*, 4 °C for 10 min to pellet mitochondria. The mitochondria were then washed twice by re-suspending in MIB with centrifugation at 11,000 × *g*, 4 °C for 10 min, weighed and re-suspended in 3 volumes of mitochondrial respiration buffer [MRB: 10 mM Tris-HCl, 25 mM KH_2PO_4, 100 mM KCl, 1 mg/ml BSA, 2 µg/ml aprotonin, pH 7.3]. The mitochondrial suspensions were kept on ice and used for respiratory experiments within 4 h of isolation.

2.3.3. Measurements of mitochondrial respiration

Protein concentrations of mitochondrial samples were measured spectrophotometrically (Spectramax Plus 384, Molecular device, Sunnyvale, CA) by the Bradford (1976) method. Mitochondrial respiration (oxygen consumption) rates were measured with Clark-type oxygen electrodes (Qubit systems, Kingston, ON). The effect of varying temperature (5, 15 and 25 °C) on CI-driven respiratory flux was tested with the assay temperature being maintained with a thermostatically controlled water circulator (Haake, Karlsruhe, Germany). This temperature range falls between the critical thermal minima and maxima for rainbow trout (Finstad et al. 1988; Rodgers and Griffiths 1983; Currie et al. 1998). Prior to all respiratory measurements, oxygen electrodes were calibrated at 0 and 100% saturation by bubbling N_2 and air to milli-Q water, respectively, at ambient atmospheric pressure (740-760 mmHg) measured by a digital barometer (Fisher Scientific, Nepean, ON). Initially, the MRB was brought to experimental temperature by placing in a water bath at the desired temperature. Subsequently, 1.45 ml MRB followed by CI substrates (5 mM malate and 5 mM glutamate) were added into the cuvette. Later, 100 µl of mitochondrial suspension containing 2-3 mg protein were introduced using Hamilton syringes and continuously stirred. The assay mixture was allowed to equilibrate and the maximal rate of mitochondrial respiration (state 3) was induced by addition of 375 nmol ADP. The state 3

eventually transitioned to basal (state 4) respiration upon depletion of the ADP. All rates of oxygen consumption were recorded and analyzed using LabPro® software (Qubit Systems) and normalized to mitochondrial protein. The effect of Cu on mitochondrial respiration was assessed using a wide range (0-2500 µM) of Cu doses (as $CuSO_4$. $5H_2O$, Sigma-Aldrich Oakville, ON). This dose regime falls within the range of Cu concentration (highest 7200 µM) measured in livers and cytosolic fractions of fish and mice (with ATP7B gene knockout) following environmental and experimental Cu exposure (Bunton et al. 1987; Garceau et al. 2010; Ralle et al. 2010; Kamunde and MacPhail 2011). The Cu doses were administered after ADP addition and Cu-impacted respiration rates were measured 3.5-4 and 8-8.5 min thereafter for state 3 and 4, respectively. State 4_{ol}, a measure of mitochondrial proton leak (Brand et al. 1994; Portner et al. 1999a) was measured 1 min after addition of 2 µg/ml oligomycin (10-10.5 min of Cu exposure). The respiratory control ratio (RCR and RCR_{ol}) and phosphorylation efficiency (P/O ratio) were then calculated as described (Chance and Williams 1955; Estabrook 1967).

2.3.4. Complex I (NADH:ubiquinone oxidoreductase) activity

Isolated mitochondria were re-suspended in MRB and 95 µl aliquots transferred into centrifuge tubes. To achieve the desired concentration of Cu (0-500 µM) in a final assay volume of 100 µl, I added 5 µl of appropriate Cu stock solution (5 µl of MRB for controls). Each exposure was done in duplicate. The mitochondria were initially incubated with Cu at 5, 15 and 25 °C for 30 min under continuous mixing following which they were washed twice by re-suspending in 500 µl of MIB with centrifugation at 10,000 × *g* for 5 min at 4 °C. The pellets obtained were re-suspended in 100 µl of MRB and used for measuring complex I activity according to method modified from Spinazzi et al. (2012).

Briefly, mitochondria were diluted to 6 mg/ml in hypotonic buffer (25 mM potassium phosphate and 5 mM $MgCl_2$) and sonicated to disrupt the IMM thus liberating the membrane bound enzyme. Subsequently, 240 µl of assay buffer (25 mM of potassium phosphate, 3.5 g/l of BSA, 0.1 mM 2,6-dichlorophenolindophenol (DCPIP), 280 µM decylubiquinone, 0.6 µg/ml antimycin A and 0.2 mM NADH, pH 7.3) and 60 µg of mitochondrial protein were added to wells of a 96-well microplate. The assays were done in triplicates for each Cu dose-temperature combination with the changes in absorbance being read at 600 nm for 5 min at 15 sec intervals (Spectramax Plus 384) without and with addition of 3 µl of 1 mM rotenone which blocks CI activity. Complex I activity was then calculated by subtracting the rotenone-insensitive activity from the total activity and converted to micromoles of DCPIP reduced using a molar extinction coefficient of 19.1 mM^{-1} cm^{-1}.

2.3.5. Mitochondrial swelling

To assess the involvement of MPTP in Cu- and temperature-induced mitochondrial dysfunction, swelling, a typical feature of MPTP (Bernardi et al. 1999), was measured spectrophotometrically (Spectramax Plus 384) according to Lee et al. (2005). Briefly, mitochondria were isolated as described above, re-suspended in MRB and their protein concentration measured by the Bradford method. The mitochondrial suspension was then diluted to 1 mg/ml with swelling buffer (100 mM KCl, 10 mM Tris-HCl, 25 mM KH_2PO_4, 1 mg/ml BSA, 2 µg/ml aprotonin, 5 mM glutamate and 5 mM malate adjusted to pH 7.3). The swelling assay was performed at room (actual 24 ^oC) temperature and 30 ^oC in a total volume of 200 µl using 0-500 µM Cu. Initially, the appropriate dose of Cu was added to the well in a volume of 20 µl. Thereafter, 180 µl of the 1 mg/ml mitochondrial suspension equilibrated to test temperature was added and the decrease in absorbance at 540 nm, indicative of mitochondrial swelling, was monitored every 10 sec for 5

min. An additional study monitored swelling induced by 200 and 500 μM Cu at 24 and 30 ºC for 60 min to assess how time affects the Cu-temperature interaction on swelling. To test if Cu-imposed swelling is due to MPTP, CsA (1 μM), a blocker of MPTP, was pre-incubated with the mitochondria for 5 min before addition of 200 μM Cu. As a positive control, Ca (100 μM) as $CaCl_2$ (Sigma-Aldrich), a known trigger of MPTP, was added to the mitochondrial suspension without and with 1 μM CsA.

2.3.6. Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was measured with a fluorescent spectrophotometer (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA) by assessing the ability of mitochondria to sequester rhodamine 123 (Rh-123), a cationic lipophilic dye (Baracca et al. 2003; Fernendez-Gomez et al. 2005). Briefly 170 μl of 1 mg/ml mitochondrial suspension and 5 μM (as 10 μl of stock solution) of Rh-123 were added to microplate wells and fluorescence (excitation 485nm, emission 528 nm) was read for 5 min to establish the baseline. Thereafter the mitochondria were energized with glutamate (5 mM) and malate (5 mM) as a 10 μl pre-mix and fluorescence was read for a further 5 min. Lastly, 200 μM Cu (as 10 μl of Cu stock solution) was added and the fluorescence was read every 16 sec for 15 min. FCCP (0.5 μM; Sigma-Aldrich), a mitochondrial uncoupler, was used as a positive control to collapse the MMP. Lastly, N-acetyl cysteine (NAC, 5 mM; Sigma-Aldrich) and 250 μM vitamin E (Sigma-Aldrich) were used to test the involvement of ROS in Cu-induced MMP changes.

2.3.7. Q_{10} calculations

The temperature sensitivity for state 3, 4 and 4_{ol} respiration rates and mitochondrial swelling (both maximal amplitude and rate) were assessed by calculating the Q_{10} values using the

equation: $Q_{10} = (R2/R1)^{[10/(T2-T1)]}$, where $R2$ and $R1$ are the mitochondria oxygen consumption rates or swelling at the respective temperatures $T2$ and $T1$ (where T2 > T1).

2.3.8. Statistical analysis

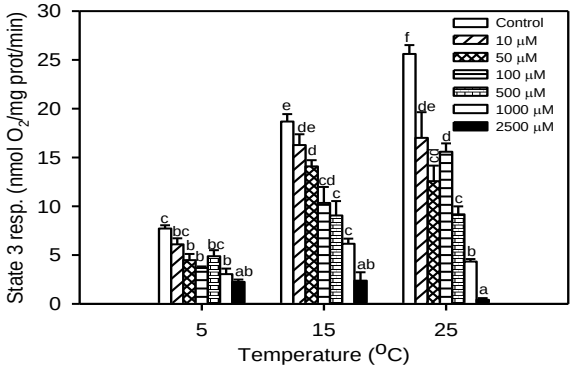
The data were checked for normality of distributions (Chi-Square test) and homogeneity of variances (Levene's test) and submitted to 2-way analysis of variance (ANOVA, Statistica StatSoft Inc., Tulsa, OK). Temperature and Cu concentration were the independent variables and *post-hoc* pair-wise comparisons were done using Tukey's honest significance difference test (HSD). The Cu doses required to inhibit maximal CI activity by 50% (IC_{50} values) were calculated using four parameter logistic non-linear regression analyses (Sigma Plot 10.0, Systat Software Inc, IL). Where applicable the data are reported as means ± SEM with a level of significance as $p < 0.05$.

2.4. Results

2.4.1. Mitochondrial respiration

Both temperature and Cu exposure affected mitochondrial respiration (Fig. 2.1). In the absence of Cu (i.e., in controls), the state 3 respiration rate (nmol O_2/mg protein/min) increased more than 3 fold from 7.7 at 5 °C to 25.6 at 25 °C. Copper exposure imposed a clear dose-dependent inhibition of maximal respiration at all temperatures with the rates decreasing by 71, 87 and 98% at 5, 15 and 25 ^{o}C, respectively, for the highest dose (Fig. 2.1b). The inhibition of maximal respiration was consistently higher at 25 °C for all the Cu doses (Fig. 2.1b) and, interestingly, Cu doses <1000 µM caused greater inhibition at 5 ^{o}C compared with 15 ^{o}C (putative control). Assessment of thermal sensitivity for maximal respiration showed that the Q_{10} values were highest for the 5-15 °C temperature range and increased for Cu doses ≤100 µM (Fig. 2.1c).

A



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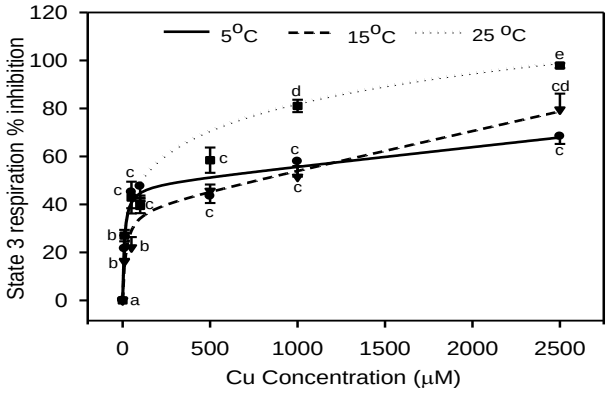


C

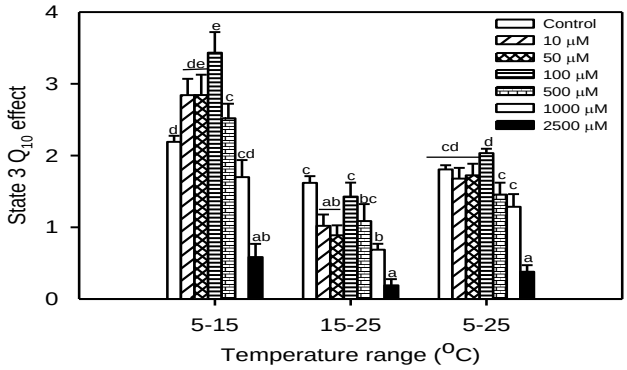


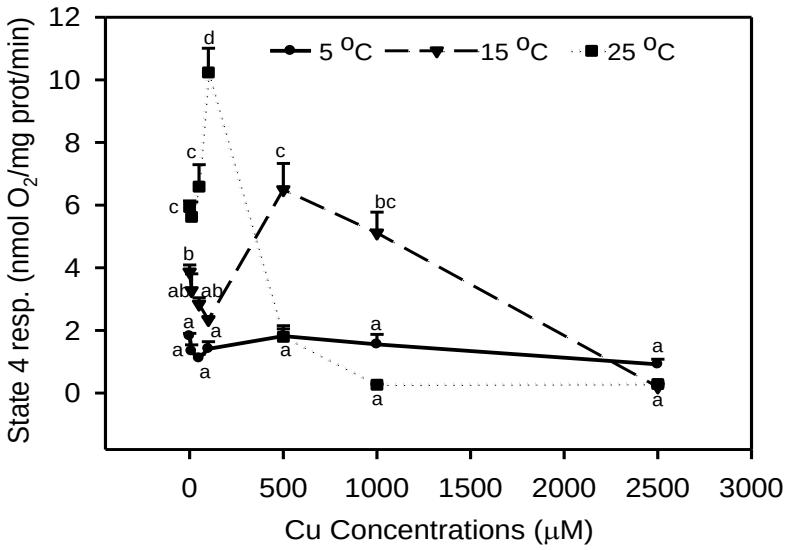
Figure 2.1. Effects of Cu and temperature on rainbow trout liver mitochondrial bioenergetic features. A: State 3 respiration; B: state 3 respiration % inhibition; C: state 3 Q_{10}. Bars or points in the same panel with different letters are significantly ($p < 0.05$) different from each other.

In contrast, Cu doses >100 μM decreased the Q_{10} values at all temperatures (Fig. 2.1c).

The state 4 in controls increased 3 fold with increasing temperature from 2 at 5 °C to 6 nmol O_2/mg protein/min at 25 °C (Fig. 2.2a). While Cu exposure did not have a significant effect at 5 °C, it imposed a biphasic response on state 4 respiration with peak stimulation at 500 μM (15 ^oC) and 100 μM (25 ^oC) and greatest inhibition at 2500 μM at all temperatures (Fig. 2.2a). The Q_{10} values in the absence of Cu were 2.5, 1.6 and 1.8 for the temperature ranges 5-15, 15-25 and 5-25 ^oC, respectively, indicating higher thermal sensitivity at low temperature (Fig. 2.2b). Generally, Cu doses ≤500 μM increased while higher doses decreased the sensitivity of state 4 respiration to temperature (Fig. 2.2b). The effects of temperature and Cu exposure on state 4_{ol} respiration (proton leak) and its thermal sensitivity mirrored those observed for state 4 respiration (Fig. 2.3).

Figure 2.4 shows the effect of temperature and Cu exposure on mitochondrial coupling efficiency (RCR). In the absence of added Cu, the RCR remained unchanged by temperature whereas effects of Cu on this metric were influenced by temperature in a complex manner. Specifically, relative to the controls, RCRs were lower for Cu doses ≥100 μM at 5 ^oC and for 500 and 1000 μM Cu at 15 ^oC. At 25 ^oC the RCR was reduced by 50,100 and 2500 μM Cu but was greatly increased by 1000 μM Cu. In contrast, RCR_{ol} decreased with temperature in the controls but similar to the effect on RCR, the response to Cu exposure depended on the dose and temperature (Fig. 2.4b). Thus, at 5 ^oC the RCR_{ol} decreased dose-dependently with Cu while at 15 ^oC only the 500 and 1000 μM Cu reduced it. The effects were more complex at 25 ^oC wherein lower (50 and 100 μM) and the high (2500 μM) doses of Cu reduced, while the mid-range dose (1000 μM) greatly increased, the RCR_{ol}.

A



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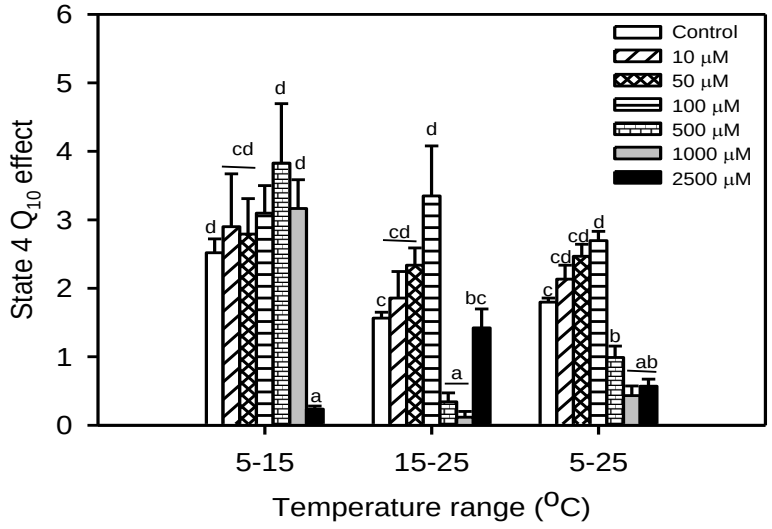
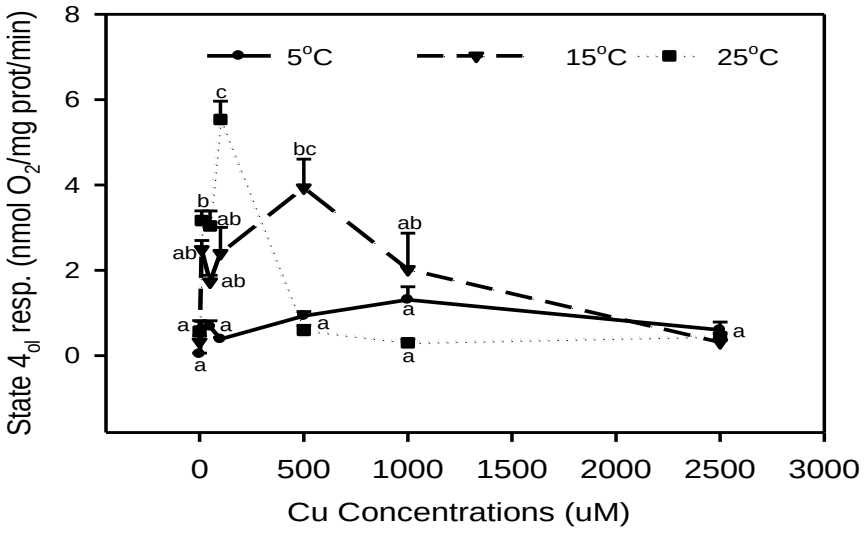


Figure 2.2. Effects of Cu and temperature on rainbow trout liver mitochondrial bioenergetic characteristics. A: State 4 respiration; B: state 4 Q_{10}. Bars in the same panel with different letters are significantly ($p < 0.05$) different from each other.

A



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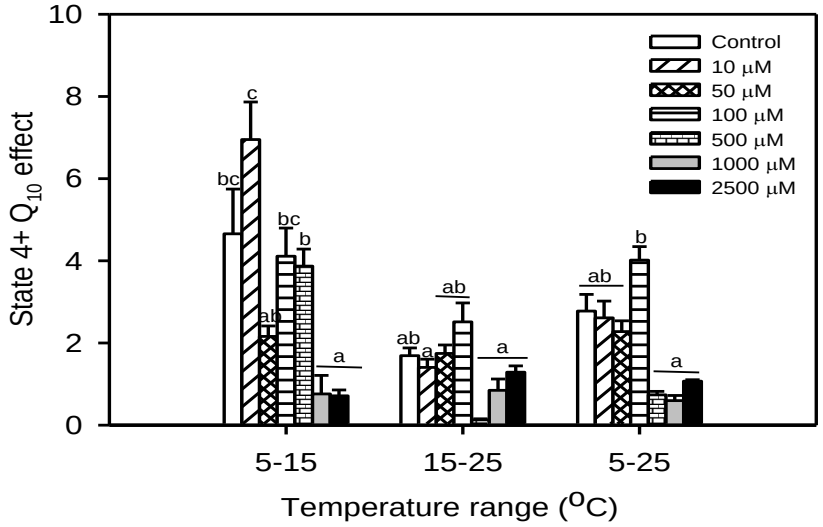
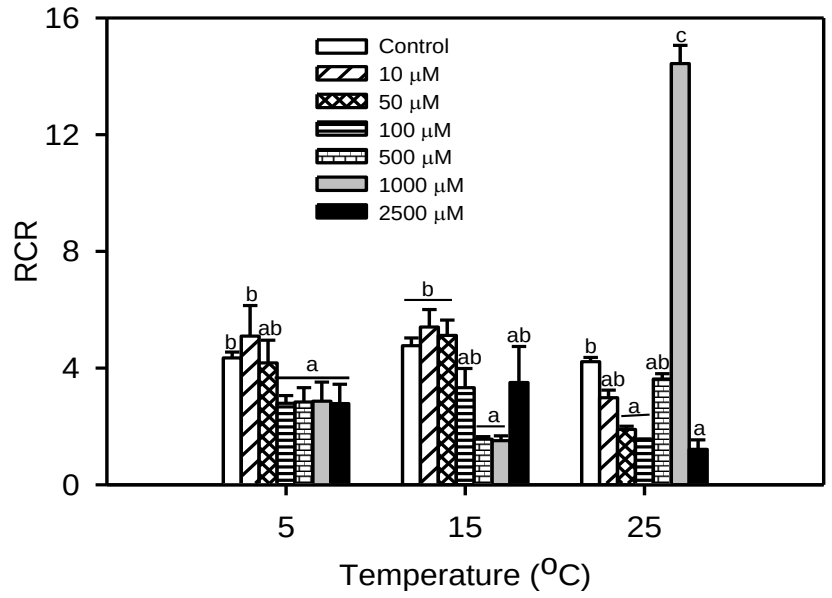


Figure 2.3. Effect of Cu and temperature on rainbow trout liver mitochondrial state 4_{ol} respiration/proton leak (A) and proton leak Q_{10} (B). Bars in the same panel with different letters are significantly ($p < 0.05$) different from each other.

A



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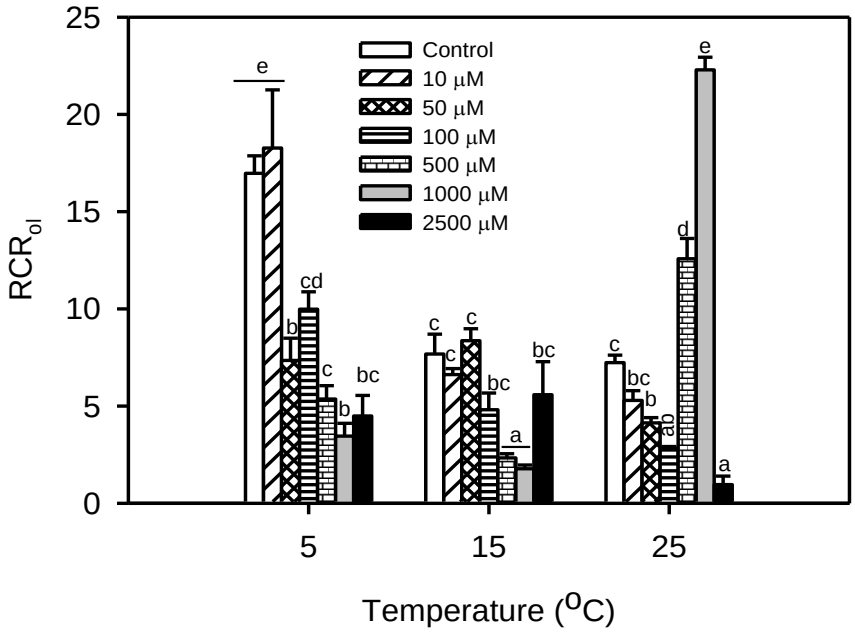


Figure 2.4. Effects of Cu and temperature on rainbow trout liver mitochondrial coupling efficiency. A: RCR; B: RCR_{ol}. Bars in the same panel with different letters are significantly ($p < 0.05$) different from each other.

The P/O ratio (Table 2.1) in control mitochondrial increased with temperature from 1.75 at 5 ^{o}C to 2.59 at 25 ^{o}C. The effect of Cu depended on the temperature with Cu doses in the range of 10-500 and 10-100 μM increasing the P/O ratios at 5 and 15 ^{o}C, respectively. Copper exposure did not affect the P/O ratio at 25 ^{o}C. The P/O ratios were not calculated for Cu doses >500 (25 ^{o}C) and >1000 (5 and 15 ^{o}C) μM because severe inhibition of state 3 respiration could not allow unambiguous demarcation of transition to state 4.

2.4.2. Complex I activity

Exposure to Cu inhibited CI activity dose-dependently whereas the incubation temperature had no effect (Fig. 2.5a). The Cu CI IC_{50}s were 240, 224 and 248 μM for incubations at 5, 15 and 25 °C, respectively (Fig. 2.5b). Note that all enzyme activity measurements were done at room temperature (24 ^{o}C).

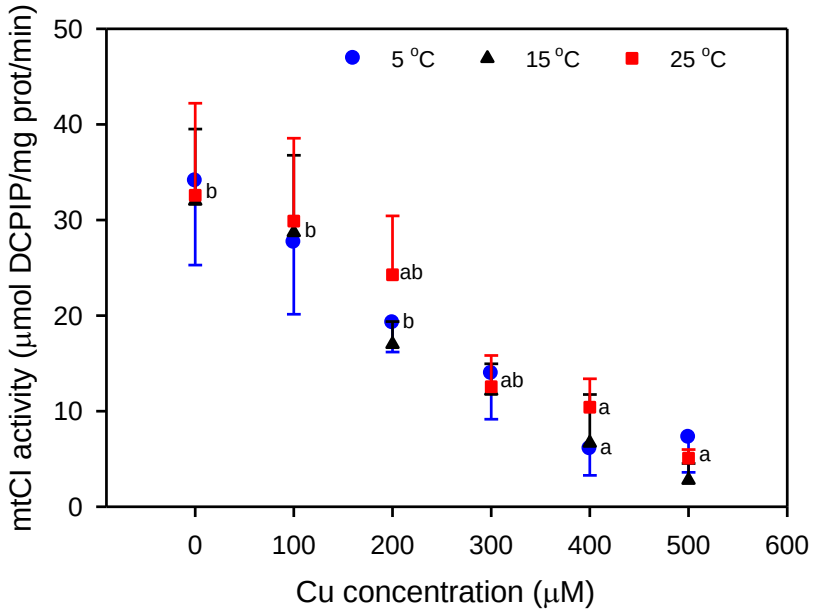
2.4.3. Mitochondrial swelling

Rainbow trout liver mitochondria exhibited Ca-induced swelling (positive control) that was completely inhibited by CsA (Fig. 2.6a,b). Due to data variability, the effect of temperature on Ca-induced swelling was not statistically significant despite the mean swelling being 30% less at 30 ^{o}C compared with 24 ^{o}C (Fig. 2.6b). All the doses of Cu tested with the exception of 500 μM at 30 ^{o}C caused mitochondrial swelling (Fig 2.7a-c) but unlike the swelling triggered by Ca, Cu-induced swelling was only partially suppressed by CsA (Fig. 2.7a,b). Moreover, the kinetics of swelling depended on the Cu dose and temperature. Thus, while spontaneous swelling was negligible over the test period, Cu-induced swelling was linear for Cu doses ≤200 μM at 24 ^{o}C but displayed an exponential decay pattern for the 500 μM Cu dose. At 30 ^{o}C, exposure to 200 μM Cu imposed exponential decay pattern of swelling while lower doses induced linear swelling.

Table 2.1. Effect of Cu and temperature on rainbow trout liver mitochondrial P/O ratios. Values with different letters are significantly different ($p<0.05$) from each other. NM indicates not measured.

Cu concentration (µM)	**Temperature (° C)**		
	5	**15**	**25**
Control	1.75 ± 0.07^{a}	1.94 ± 0.06^{ab}	2.59 ± 0.07^{c}
10	2.51 ± 0.08^{c}	2.65 ± 0.13^{c}	2.67 ± 0.13^{c}
50	3.92 ± 1.43^{d}	2.65 ± 0.15^{c}	2.61 ± 0.22^{c}
100	2.69 ± 0.09^{c}	2.43 ± 0.21^{c}	2.40 ± 0.15^{c}
500	3.45 ± 0.61^{d}	1.99 ± 0.14^{b}	NM
1000	NM	NM	NM
2500	NM	NM	NM

A



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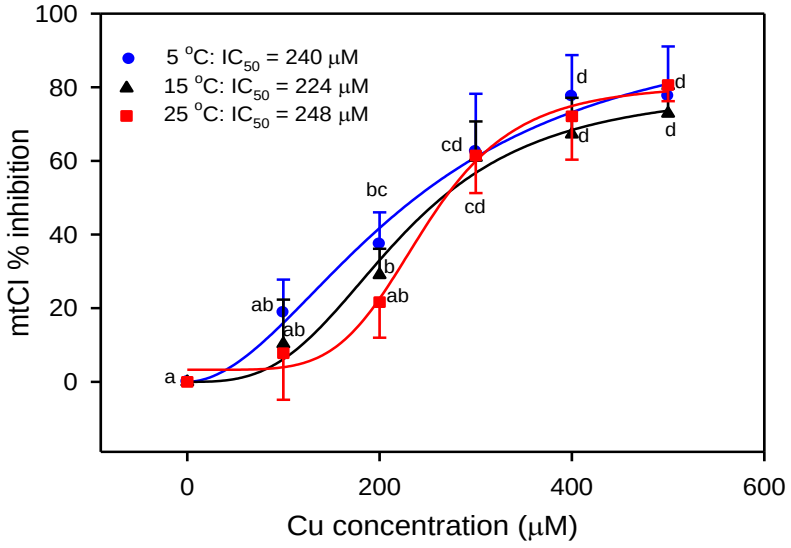
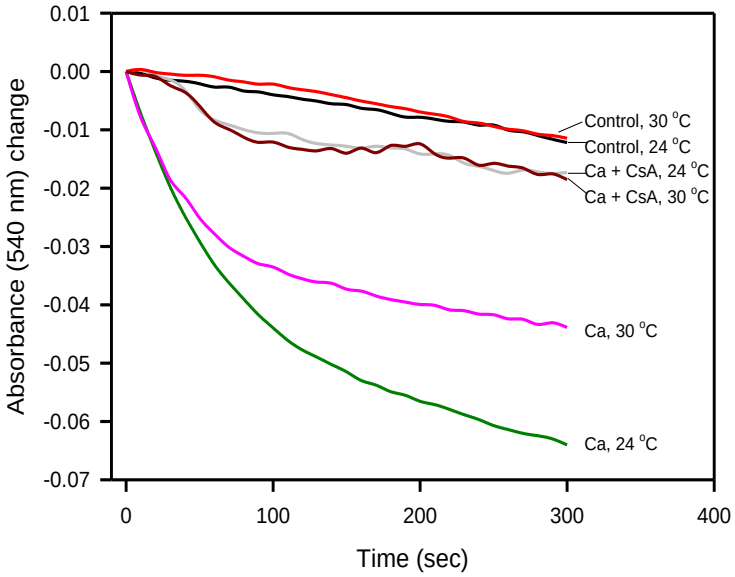


Figure 2.5. Effects of Cu and temperature on rainbow trout liver mitochondria complex I activity. A: complex I activity following 30 min incubation with Cu at 5, 15 and 25 °C; B: Complex I Cu ED_{50}. Points with different letters are significantly ($p < 0.05$) different from each other.

A



B

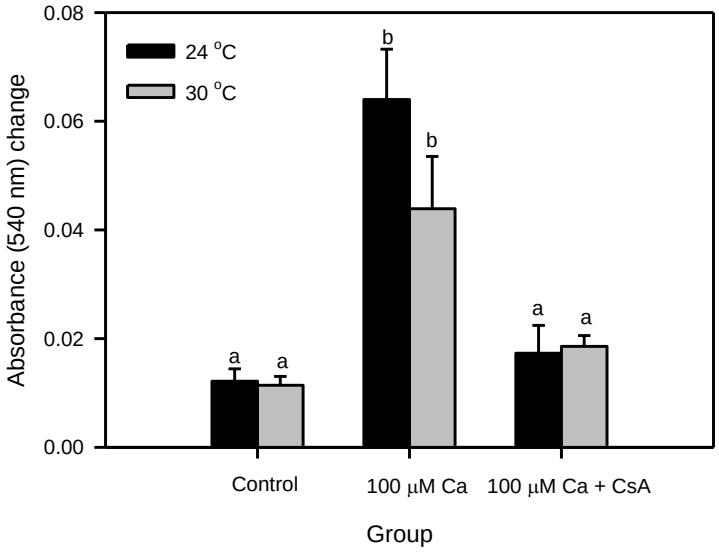


Figure 2.6. Rainbow trout liver mitochondrial swelling positive control (Ca-induced and reversed by cyclosporine A). A: trend-lines of mean changes in absorbance at 540 nM for mitochondria of 6 fish at 24 and 30 °C; B: means ± SEM maximal swelling after 5 min at 24 and 30 °C. Bars with different letters are significantly different from each other, $p < 0.05$.

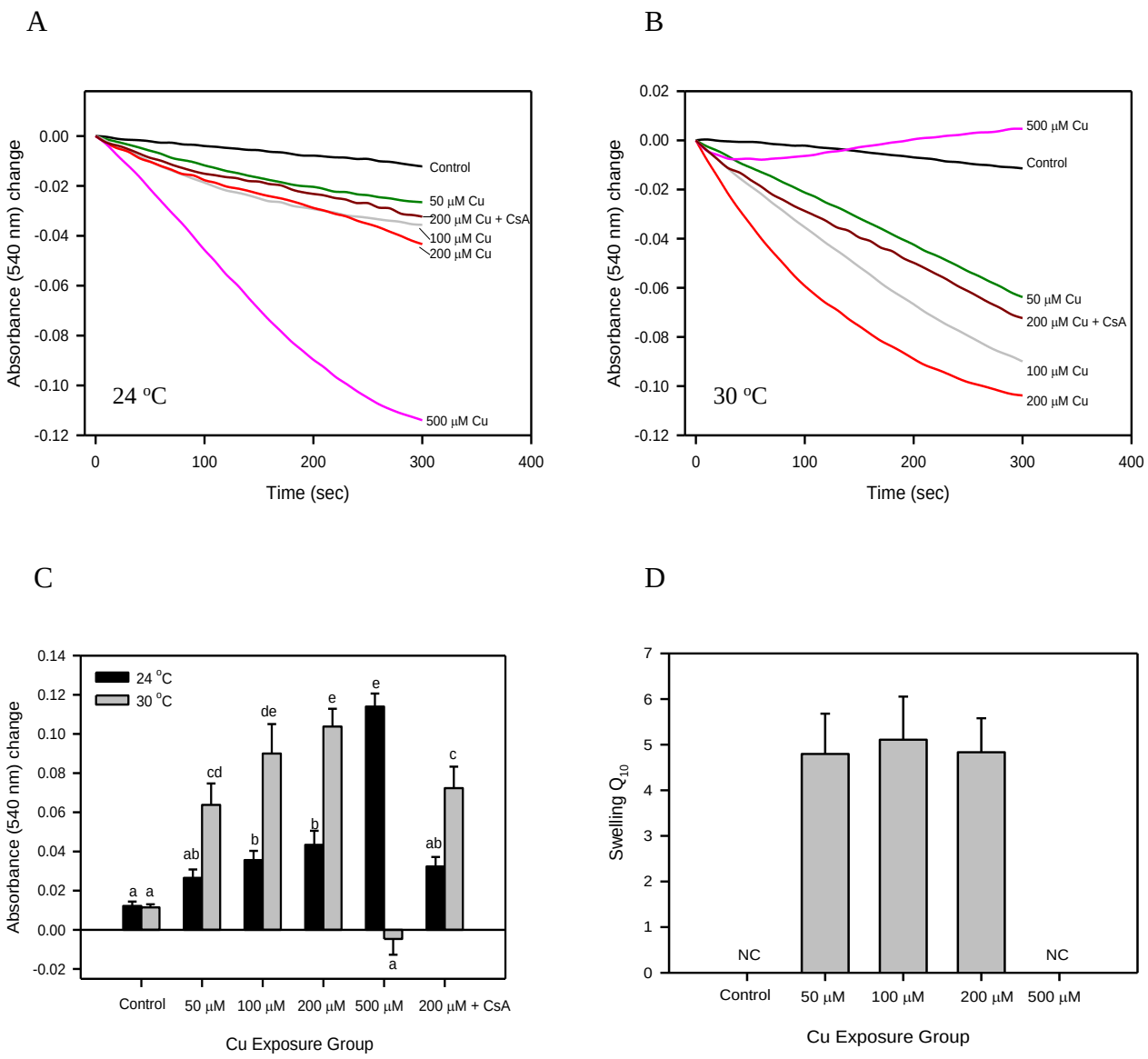


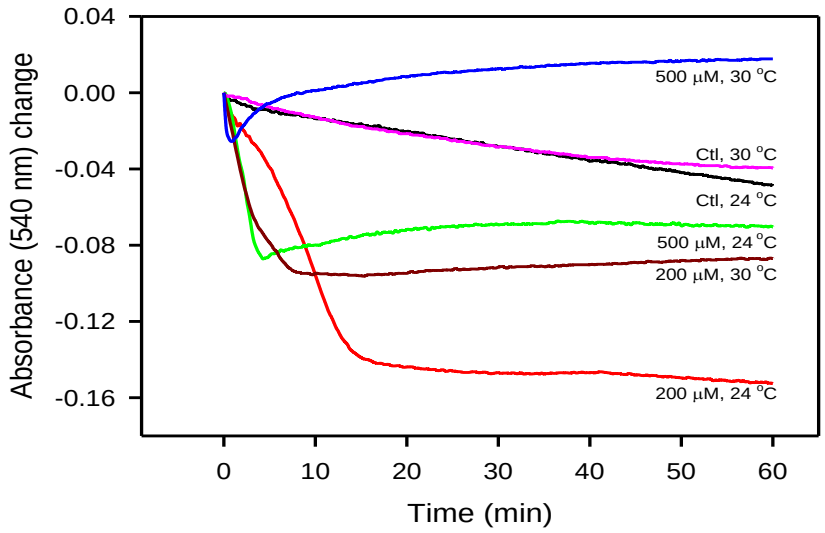
Figure 2.7. Effects of temperature and Cu on rainbow trout liver mitochondrial swelling. A: trend-lines of mean changes in absorbance at 540 nM for mitochondria of 6 fish at 24 °C; B: trend-lines of mean changes in absorbance at 540 nM for mitochondria of 6 fish at 30 °C; C: means ± SE maximal swelling after 5 min at 24 and 30 °C; D: means ± SEM Q_{10} values for Cu-induced swelling. Bars with different letters are significantly different from each other, $p < 0.05$.

Intriguingly, 500 μM Cu caused mild mitochondrial contraction (increased absorbance) at 30 ºC. Although temperature had no effect on spontaneous swelling, Cu-induced swelling was highly sensitive to temperature with the Q_{10} values calculated over 24-30 ºC range averaging about 5 for doses ≤200 μM (Fig. 2.7d). Finally, swelling measurements for the two highest Cu doses over 1 h revealed that high temperature caused mitochondria to contract and attain maximal swelling faster (Fig. 2.8A). Moreover, similar to swelling amplitude, swelling rates exhibited high thermal sensitivity with Q_{10} values of 4.5 (200 μM) and 7.2 (500 μM) (Fig. 2.8B).

2.4.4. Membrane potential

The stabilization of mitochondria following transfer from ice (4 ºC) directly to the assay temperatures (24 and 30 ºC) was characterized by increased fluorescence (Figs. 2.9 and 2.10). In contrast, mitochondrial energization with malate and glutamate caused the fluorescence to decrease due to sequestration of Rh-123 in mitochondrial matrix driven by the generated membrane potential (inside negative) in resting mitochondria (Figs. 2.9 and 2.10). Un-energized mitochondria exhibited no change in fluorescence and as expected, addition of the uncoupler FCCP caused a huge upswing in fluorescence indicative of membrane potential dissipation. Similarly, the addition of 200 μM Cu caused an increase in fluorescence comparable to that evoked by FCCP. The Cu-imposed dissipation of MMP was clearly reversed by NAC (Figs. 2.9 and 2.10) and vitamin E (Fig. 2.11). There was no difference in the amplitude of swelling between the measurements made at 24 (Fig. 2.9) and 30 (Fig. 2.10) ºC except that NAC significantly minimized the MMP loss imposed by 4→30 ºC temperature shock.

A



B

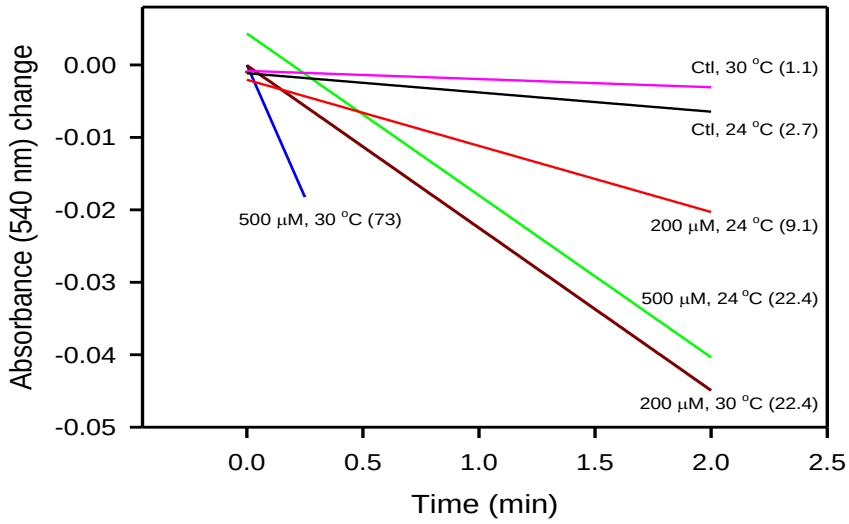


Figure 2.8. Kinetics of rainbow trout liver mitochondrial volume changes at 24 °C and 30 °C. A: maximal amplitude of swelling after 60 min; B: maximal rates of swelling measured for the initial linear phase (2 min except for the 500 µM Cu at 30 °C which is 15 sec). Numbers in brackets in panel B indicate swelling rates in absorbance milliunits/min × 10^3. Ctl = control. Each trend-line represents average of 4 readings from mitochondria of 2 fish.

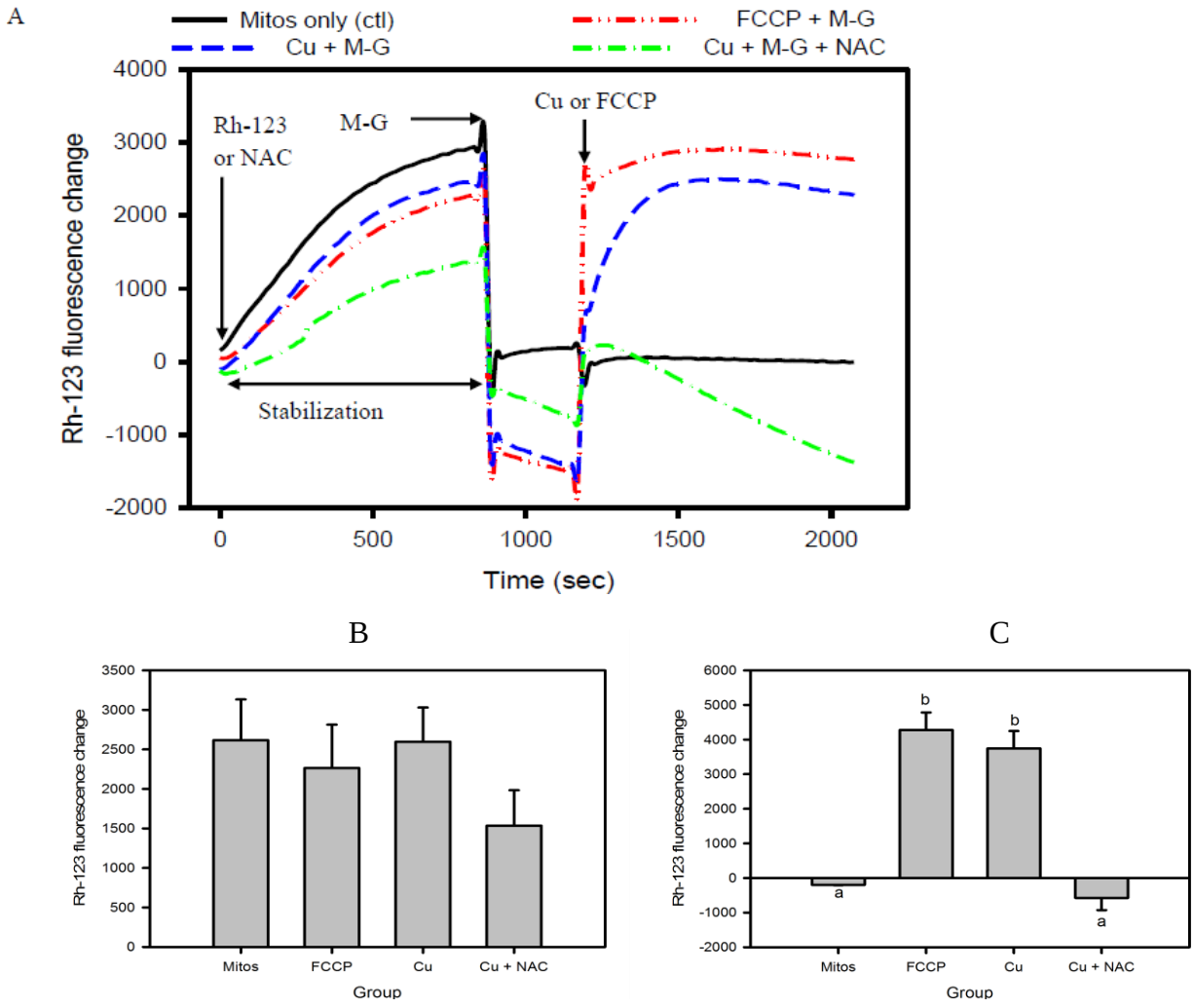


Figure 2.9. Effects of Cu (200 μM), energization and temperature shock (4→24 ºC) on rainbow trout liver mitochondrial membrane potential (MMP). FCCP (0.5 μM) was used as a positive control to collapse the MMP and N-acetyl cysteine (NAC; 5 mM) was used to test the potential role of reactive oxygen species (ROS) in Cu-induced MMP changes. A: kinetics of MMP changes showing stabilization period and points of addition of Rhodamine 123 (Rh-123; 5 μM), malate and glutamate (M-G; 5 mM), NAC, and Cu or FCCP. Mitos = mitochondria. B: amplitude of fluorescence change due to temperature shock. C: amplitude of fluorescence change due to modulators. Trend-lines in A are mean values of readings from mitochondria prepared separately from livers of 5 fish. Data in B are means ± SEM (n = 5) of the difference in fluorescence from the start of the run till MMP stabilized (after 15 min). Data in C are means ± SE (n = 5) of the difference in fluorescence from point of addition of M-G to the end of the run.

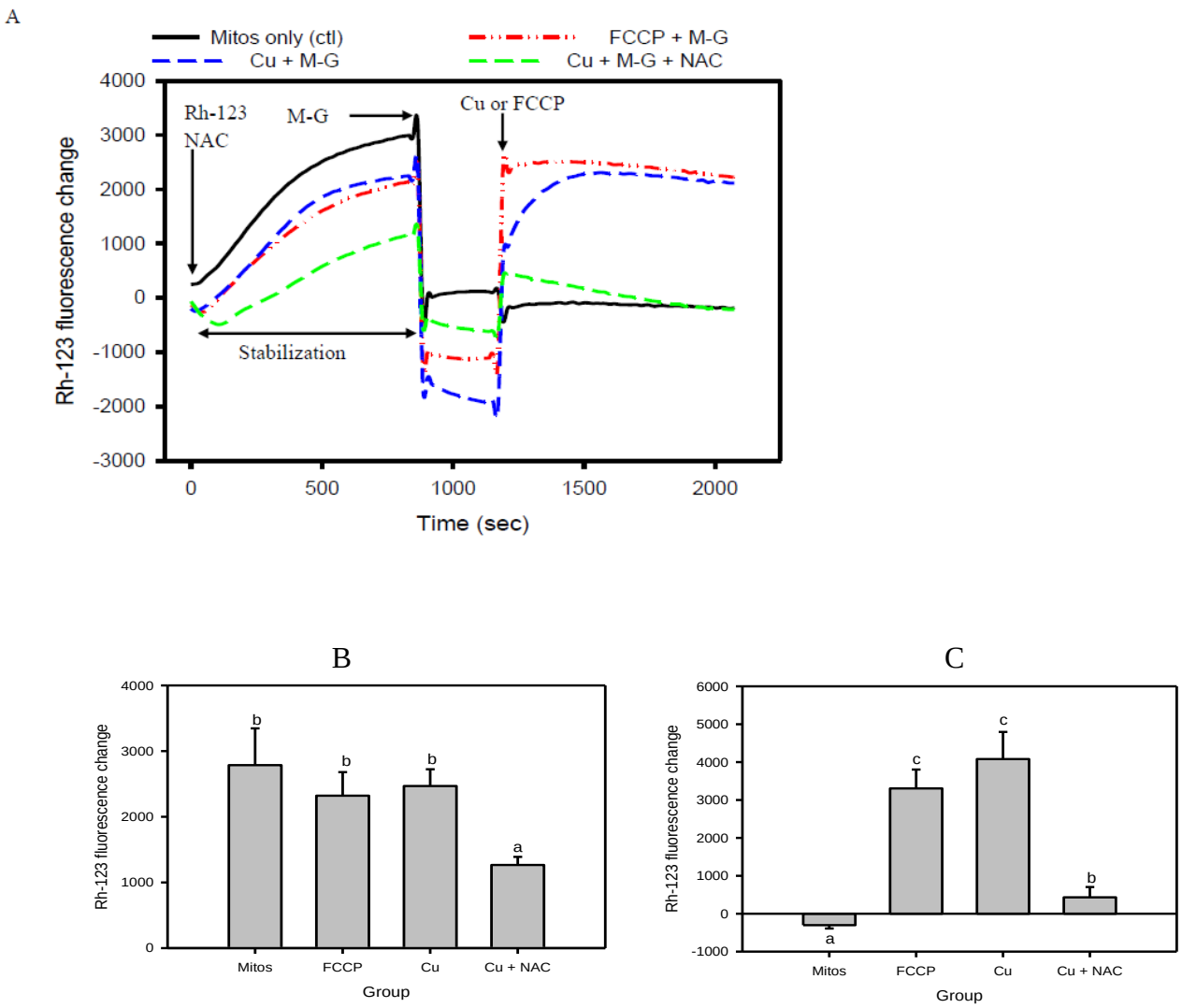


Figure 2.10. Effects of Cu (200 μM), energization and temperature shock (4→30 ºC) on rainbow trout liver mitochondrial membrane potential (MMP). FCCP (0.5 μM) was used as a positive control to collapse the MMP and N-acetyl cysteine (NAC; 5 mM) was used to test the potential role of reactive oxygen species (ROS) in Cu-induced MMP changes. A: kinetics of MMP changes showing stabilization period and points of addition of Rhodamine 123 (Rh-123; 5 μM), malate and glutamate (M-G; 5 mM), NAC, and Cu or FCCP. Mitos = mitochondria. B: amplitude of fluorescence change due to temperature shock. C: amplitude of fluorescence changes due to modulators. Trend-lines in A are mean values of readings from mitochondria prepared separately from livers of 5 fish. Data in B are means ± SEM (n = 5) of the difference in fluorescence from the start of the run till MMP stabilized (after 15 min). Data in C are means ± SE (n = 5) of the difference in fluorescence from point of addition of M-G to the end of the run.

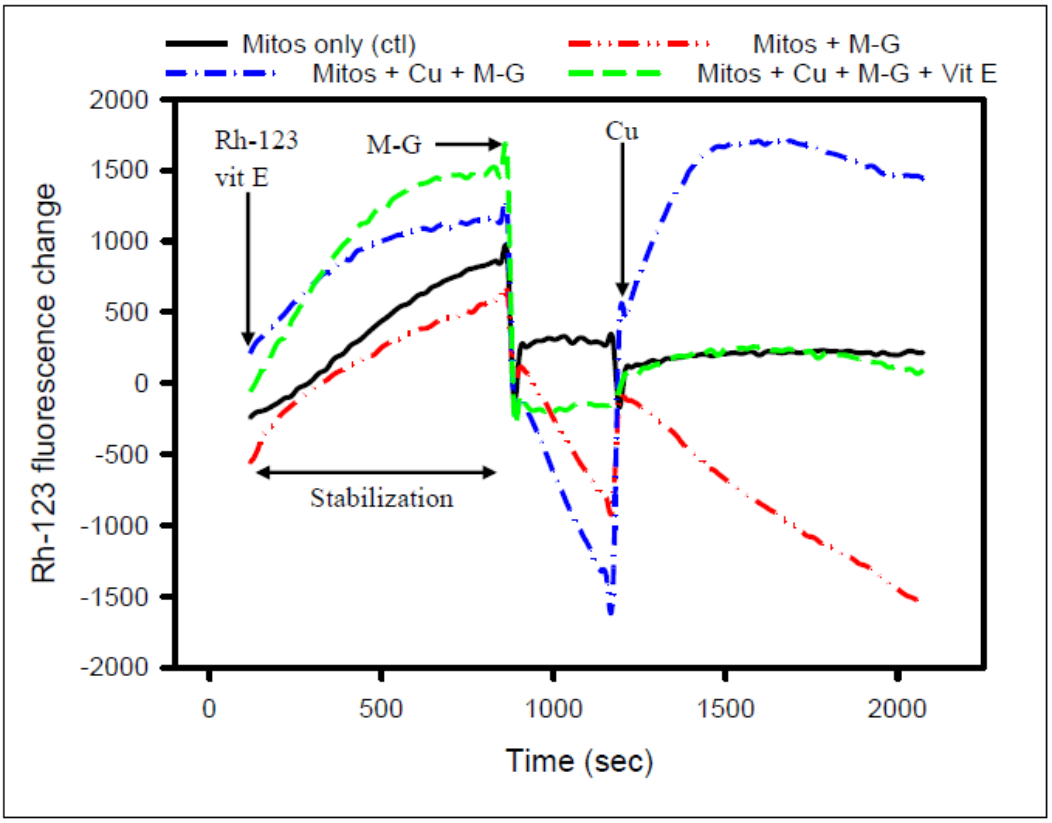


Figure 2.11. Effects of Cu, vitamin E, energization and temperature shock (4→24 ^{o}C) on rainbow trout liver mitochondrial membrane potential (MMP). Vitamin E was used to confirm the role of ROS in Cu-induced MMP changes. The kinetics of MMP changes showing stabilization period and points of addition of Rhodamine 123 (Rh-123; 5 μM), malate and glutamate (M-G; 5 mM), vitamin E (250 μM) and Cu (200 μM) are presented. Mitos = mitochondria. Trend-lines in are mean values of duplicate readings from mitochondria prepared separately from livers of 3 fish.

2.5. Discussion

2.5.1. Effects of Cu and temperature on mitochondrial bioenergetics

The present study investigated Cu-temperature interactions on mitochondrial bioenergetics to provide mechanistic insights into how temperature affects Cu sensitivity of mitochondrial bioenergetics thereby shedding light on potential effects of naturally- and anthropogenically-induced temperature fluctuations on cellular/organismal energy homeostasis. The results obtained show that the interactive responses of Cu and temperature stress on mitochondrial function are complex and depend on the Cu dose, temperature and the response assessed. Over the range tested, temperature profoundly stimulated the maximal mitochondrial respiration in accord with several other studies in ectotherms (Abele et al. 2002; Keller et al. 2004; Portner 2002) and endotherms (Willis et al. 2000; Lemieux et al. 2010; Zukiene et al. 2010). While it is tempting to conclude that high maximal respiration indicates increased ATP synthesis, prudence is warranted because a high respiration rate at elevated temperature may be due to increased proton leak, which does not contribute to ATP synthesis (Pörtner et al. 1999; Sommer et al. 1997). Because I did not observe inhibition of maximal respiration at 25 ^{o}C as would be expected at unfavorably high temperature (Zukiene et al. 2010; Guderley 2011; Iftikar and Hickey 2013; Blier et al. 2014) I concluded that under the experimental conditions employed, this temperature level alone did not constitute sufficient stress to impair CI-driven respiration in rainbow trout liver isolated mitochondria. Copper exposure on the other hand inhibited the maximal respiration in agreement with previous studies in rats (Saris and Skulskii 1991; Belyaeva et al. 2004, 2011), rainbow trout (Sappal et al. 2014b) and bivalves (Collins et al. 2010; Ivanina and Sokolova 2013). The inhibition of maximal respiration was consistently greater at higher temperature and interestingly, lower Cu doses caused greater inhibition at 5 ^{o}C compared with the putative control (15 ^{o}C). This indicates that both high

and low temperatures exacerbate the deleterious effects of Cu, possibly due to a surge in ROS generation attributable to inhibition of the ETS and/or changes in membrane fluidity at temperature extremes (Zukiene et al. 2010; Guderley 2011; Moyes and Ballantyne 2011; Blier et al. 2014). The Q_{10} for maximal respiration was highest at lower temperatures with low Cu doses increasing and higher doses decreasing it. Although high Q_{10} values at lower temperatures are frequently reported for maximal mitochondrial respiration (Guderley and Johnston 1996; Abele et al. 2002; Lemieux et al. 2010), a consistent theme remains elusive because the converse has also been reported (Chamberlin 2004). Overall, regarding interactive effects, the present findings and those from other studies (Sokolova 2004; Cherkasov et al. 2006; Sappal et al. 2014b) suggest that temperature sensitizes mitochondrial maximal respiration to metals.

The basal/state 4 respiration increased with increasing temperature consistent with previous studies (Hardewig et al. 1999a; Abele et al. 2002; Lemieux et al. 2010) and as observed with maximal respiration, the Q_{10} values were greater at low temperature. Copper exposure imposed a biphasic response on basal respiration characterized by low dose stimulation, with the doses inducing the maximum stimulation becoming progressively reduced with increasing temperature (left shift of peaks in Fig. 2.2A) indicating increased sensitivity to Cu. A review of the available literature on the effect of Cu on state 4 respiration in malate-glutamate-energized liver mitochondria showed inconsistent results with both lack of effect (Belyaeva et al. 2011) and stimulation (Saris and Skulskii 1991) being reported. The present study explains this discrepancy by showing that a range of effects is possible depending on the Cu dose used.

Because state 4 respiration is primarily controlled by proton leak (Brand and Nicholls, 2011) I estimated this leak by measuring mitochondrial respiration in the presence of oligomycin (state 4_{ol}), an inhibitor of CV. I found that state 4_{ol} increased with temperature in agreement with

previous studies (Brookes et al. 1998; Hardewig et al. 1999a). Importantly, proton leak exhibited high thermal sensitivity and constituted an increasingly greater proportion of respiration at high temperatures indicative of reduced OXPHOS efficiency. Regarding interacting effects, Cu at low doses acted synergistically with high temperature to exacerbate proton leak. Mechanistically, the increased proton leak observed here could result from temperature-induced changes in membrane permeability and/or activity of the proteins that mediate this process (Moyes and Ballantyne 2011; Fields 2011; Hazel 1995). Copper also can modify these proteins and membranes through oxidative stress and binding to thiols (Garcia et al. 2000; Letelier et al. 2005; Zischka and Lichtmannegger 2014) as well as by inducing MPTP (Belyaeva 2011; Reddy et al. 2008; Zischka and Lichtmannegger 2014; this study), thereby increasing proton leak.

To assess the overall impact of temperature and Cu, I calculated the RCR and P/O ratios, two classical indicators of mitochondrial dysfunction influenced by virtually all functional features of OXPHOS (Brand and Nicholls, 2011). I found no temperature-induced alteration in RCR because state 3 and 4 rates of respiration had similar thermal sensitivity and increased proportionately over the temperature range tested. While my findings are in accord with those of Guderley and Johnston (1996) for rainbow trout muscle mitochondria, they contrast with several other studies using mitochondria from endotherms (Brooks et al. 1971; Dufour et al. 1996; Lemieux et al. 2010; Zukiene et al. 2010) and ectotherms (Hardewig et al. 1999a; Abele et al. 2002; Keller et al. 2004; Hilton et al. 2010; Iftikar and Hickey 2013). All of the latter studies reported that beyond a species-specific critical temperature the RCR decreased, thus lending support to the notion that the highest temperature (25 ^{o}C) tested in the present study was well-tolerated by rainbow trout liver mitochondria. Copper exposure on the other hand induced varying effects ranging from absence of effect to decreased or increased RCR depending on the temperature-Cu dose combination,

which is consistent with my recent study on CII-driven respiratory flux (Sappal et al. 2014b). These dissimilar changes in coupling can be explained by the inhibitory effect of high Cu and stimulatory effect of high temperature on maximal respiration, the stimulatory effect of low doses of Cu and high temperature on basal respiration, and the inhibitory effect of high Cu doses on basal respiration. Regardless of the cause of the varied results here, there does appear to be a common thread that sufficiently high temperature and Cu doses reduce mitochondrial coupling efficiency. Lastly, it is noteworthy that Sappal et al. (2014b) observed a clear increase in RCR at 25 ^{o}C for the mid-range Cu doses similar to the present study (Fig. 2.3). These results together with the absence of swelling at a comparable Cu-temperature combination (Fig. 2.7), suggest that substantial and unique mitochondrial changes occur *in vitro* at this combination of the two stressors.

The P/O ratio increased with temperature in agreement with Lemieux et al. (2010) although several studies (Brooks et al. 1971; Dufour et al. 1996; Abele et al. 2002; Sokolova 2004) have reported that the P/O decreases with temperature. These discrepancies likely result from differences in experimental conditions or animal species used, particularly the temperature range, among studies. Although I found that low Cu doses increased the P/O ratio at lower temperatures, Sokolova (2004) and Adiele et al. (2010, 2012) reported unchanged or reduced P/O ratios following exposure of oyster gill and rainbow trout liver mitochondria to Cd, respectively. Overall, all studies to date indicate that the utility of P/O ratio in assessing the effects of metals on OXPHOS is constrained by the inability to demarcate state 4 transitions at higher metal doses. Nonetheless, the finding that failure to transition to state 4 occurs at lower doses of Cu at high temperature (Table 2.1) supports the theme that temperature sensitizes mitochondria to Cu toxicity.

2.5.2. Effect of Cu and temperature on mitochondrial complex I activity

Following the finding that Cu inhibits uncoupler-stimulated respiration, which is indicative of ETS impairment, I identified that Cu directly inhibits CI enzyme activity. Mitochondrial CI inhibition not only reduces the respiratory flux through the ETS but also increases ROS generation (Turrens 2003; Murphy 2009). Because Cu in and of itself induces ROS generation (Linder and Hazegh-Azam 1996; Ferretti et al. 2003), augmented mitochondrial ROS generation through the process of "ROS-induced ROS" (Li et al. 2013) is a likely sequel of Cu exposure. While ROS generation was not directly measured, I demonstrate that MMP dissipation by Cu is reversed by the ROS scavengers NAC and vitamin E (Figs. 2.8, 2.10 and 2.11). Thus, the present study including the demonstration that CII is also inhibited by Cu (Sappal et al. 2014b), suggests that Cu targets molecular components common to CI and II. These molecular components are possibly thiols, which are prevalent in the mitochondrial ETS enzymes and are known Cu targets (Verity and Gambell 1968; Wojtczak et al. 1996).

Surprisingly, the hypothesis that Cu would have lesser impact on CI activity when the exposures were done at lower compared with higher temperatures was not borne out. It is likely that under my experimental conditions the resultant metal-enzyme interactions were not sufficient to induce discernable effects when enzyme activity assay was performed at a common temperature. Notably, my findings contrast those of Ivanina et al. (2008) who found a clear temperature dependence of CI sensitivity to Cd in eastern oyster mitochondria, albeit with enzyme activity measurements at different temperatures.

2.5.3. Effect of Cu and temperature on mitochondrial swelling

The induction of mitochondrial swelling by Ca and its attenuation by CsA confirms the presence of a canonical MPTP in rainbow trout liver mitochondria. Importantly, I show for the first time using swelling assay in isolated rainbow trout mitochondria that Cu exposure induces MPTP opening in this species. Although an earlier study using rainbow trout hepatocytes (Krumschnabel et al. 2005) did not detect overall MPTP induction (assessed by membrane potential measurement) following Cu exposure, a subpopulation of the cells did express this phenomenon thus affording support to my findings. In the rat, Reddy et al. (2008) observed Cu-induced CsA-sensitive mitochondrial swelling while others (Belyaeva et al. 2004; 2011; Saris and Skulskii 1991; Wojtczak et al. 1996) did not. Because CsA only partly prevented Cu-induced swelling here, other mechanisms in addition to MPTP are involved in rainbow trout mitochondrial swelling. These other mechanisms are possibly mediated by K^+ because the concentration of this cation is much higher in cytosol/buffer relative to the mitochondrial matrix (Kaasik et al. 2007) and its influx into the mitochondria would be accompanied by osmotically obliged water resulting in swelling. It is possible that Cu promotes the opening of mitochondrial K^+ channels because the exposure of this metal to rat liver mitochondria caused K^+ uptake (Zaba and Harris, 1976; Wojtczak et al. 1996). Lastly, binding of Cu to thiols, e.g., those of ANT, a core protein partner of MPTP, can stimulate pore opening with subsequent K^+ and water influx (Halestrap et al. 1997; Garcia et al. 2000).

The shift from linear to hyperbolic pattern of mitochondrial swelling with Cu dose suggests that swelling is influenced by the inducing conditions. However, this shift was constrained by temperature to 200 µM Cu because a higher Cu dose (500 µM) caused mild contraction instead of swelling at 30 ^{o}C. In accord with my findings, Verity and Gambell (1968) reported different patterns (biphasic and sigmoidal) of swelling dependent on the Cu dose in rat liver mitochondria.

The mechanisms underlying the complex Cu-induced mitochondrial swelling kinetics observed here remain unclear but could in part be related to the composite mitochondrial structure which potentially can allow different phases of swelling particularly if accompanied by uneven temperature-induced changes in membrane permeability/fluidity. Other probable explanations consistent with the present results include (i), direct inner membrane depolarization by Cu and high temperature (Fig. 2.8) or as a result of inhibition of ETS activity at high Cu (Fig. 2.1 and 2.4) and (ii), inhibition of MPTP formation at high Cu and high temperature (Fig. 2.7).

The observation that temperature did not affect spontaneous and Ca-induced mitochondrial swelling but profoundly influenced Cu-evoked swelling is intriguing and suggests that the two cations cause swelling by different mechanisms. In this regard, the high thermal sensitivity (Q_{10} ~5) of Cu-induced swelling suggests that it is a biochemical as opposed to a purely physical process, e.g., diffusion. Additionally, membranes can present an appreciable barrier to the diffusion of charged solutes resulting in high Q_{10} values (Nobel 2009) but this still does not explain my results because both Cu and Ca are charged. It is also possible that Cu-induced swelling is a non-enzymatic/spontaneous reaction because these reactions also bear high Q_{10} values (Elias et al. 2014). Irrespective of the underlying mechanisms, Verity and Gambell (1968) reported comparable Q_{10} values (~4) for Cu-induced swelling in rat liver mitochondria indicating that this phenomenon may be typical of mitochondria exposed to Cu. Collectively, temperature modulates Cu-induced swelling and high Cu-high temperature combination appears to impose alterations in mitochondrial membrane properties or ion fluxes that inhibit mitochondria swelling and promote contraction. We speculate that inhibition of ETS and dissipation of MMP at these conditions (Figs 2.1, 2.4 and 2.7) impedes K^+ influx, ultimately causing K^+ to equilibrate across the IMM and blocking swelling. Additionally, the mitochondrial contraction possibly resulted from activation

of K^+/H^+ antiporter following K^+ and water accumulation in the matrix (Zaba and Harris, 1976; Garlid and Paucek 2001; Lee et al. 2005).

2.5.4. Effect of Cu and temperature on membrane potential

Being the dominant component of the mitochondrial proton motive force, MMP constitutes the primary driving force for ATP synthesis (Dimroth et al. 2000). Indeed, MMP is arguably the epicenter of OXPHOS because it serves as the connecting intermediary of mitochondrial subsystems (Dufour et al. 1996; Chamberlin 2004; Zukiene et al. 2010) whose alteration has implications for the global mitochondrial function. Consistent with previous studies (Zukiene et al. 2010; Iftikar and Hickey 2013) I found that temperature rise reduces MMP. Further, unlike Belyaeva et al. (2011) who found negligible effects of Cu on MMP in CI energized rat liver mitochondria, here Cu caused a marked downswing in MMP comparable to that caused by the uncoupler FCCP (Figs. 2.8 and 2.10) suggesting that Cu was electrically transported into the mitochondrial matrix or that it acted as an uncoupling agent. Alternative explanations here are that the MMP collapse resulted from increased Cu-induced K^+ influx as showed in rat mitochondria (Zaba and Harris 1976; Wojtczak et al. 1996), inhibition of the ETS or opening of MPTP. Importantly, Cu-induced MMP dissipation was inhibited by NAC and vitamin E suggesting involvement of ROS in this response. Note that while a strong positive correlation between MMP and ROS generation has repeatedly been observed (Suski et al., 2012) increased ROS production at low MMP has also been reported (Zamzami et al. 1995; Nicholls and Ward 2000; Murphy, 2009; Lebiedzinska et al. 2010).

2.6. Conclusions

Rainbow trout liver mitochondria are highly responsive to temperature and although the RCR was unchanged, the P/O ratio increased with temperature suggesting that mitochondrial efficiency improved over the temperature range tested. Copper impaired mitochondria function by inhibiting the respiratory flux through CI, stimulating membrane permeability in part by inducing MPTP, stimulating proton leak and dissipating the MMP. While temperature sensitized mitochondria to Cu toxicity leading to greater dysfunction at both low and high temperature, some combinations of the two stressors inhibit swelling and improve coupling. Overall, my findings indicate greater bioenergetic disturbances during concurrent Cu and thermal stress but drawing general conclusions about interactive effects on a multi-component system like mitochondria is difficult because individual components do not always respond in the same way.

CHAPTER 3

COPPER ALTERS THE EFFECT OF TEMPERATURE ON MITOCHONDRIAL BIOENERGETICS IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

A version of this Chapter has been published as:

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3.1. Abstract

I investigated interaction of temperature and Cu on mitochondrial bioenergetics to gain insight into how temperature fluctuations imposed by natural phenomena or anthropogenic activities would modulate the effects of Cu on cellular energy homeostasis. Mitochondria were isolated from rainbow trout livers and, in the first set of experiments, exposed to Cu (0-2.5 mM) at 5, 11, and 25 ^{o}C with measurement of mitochondrial CII-driven respiration. In the second set of experiments, un-energized mitochondria were incubated for 30 or 60 min with lower concentrations (0-160 μM) of Cu to measure the effects on CII enzyme activity. Whereas maximal/state 3 respiration was inhibited by high Cu exposure, low Cu doses stimulated and high Cu doses inhibited resting/state 4 and 4_{ol} /proton leak respirations. High temperature alone increased mitochondrial respiration in all states. The Q_{10} values for state 3, 4 and proton leak respirations suggested active processes with state 4 respiration and proton leak exhibiting greater thermal sensitivity than state 3 respiration. The differential thermal sensitivity of resting relative to phosphorylating mitochondrial state led to uncoupling and limitation of mitochondrial oxidative capacity at both high temperature and doses of Cu. Moreover, exposure to high Cu caused loss of thermal dependence of mitochondrial bioenergetics culminating in Q_{10} values well below unity and reduced activation energies (E_a) for both maximal and resting respiration rates. Additionally, CII activity was increased by low and reduced by high doses of Cu indicating that direct effects on this enzyme contribute to Cu-induced mitochondrial dysfunction. Taken together, it appears that the substrate oxidation (ETS and TCA) and proton leak subsystems are targets of deleterious effects of Cu and elevated temperature on mitochondrial bioenergetics. However, mitochondrial effects of Cu and temperature may not be easily distinguished because they are generally qualitatively similar.

3.2. Introduction

High levels of trace metals and adverse temperatures represent two widespread natural and anthropogenically driven stress factors that pose a significant threat to the integrity of aquatic systems (Sala et al. 2000; Christensen et al. 2006; Altshuler et al. 2011). While these stressors are known to affect diverse biological processes in aquatic organisms, disturbance in energy homeostasis is perhaps their most important adverse effect due to the pervasive influence of energy availability on fitness and survival. Although significant progress has been made towards understanding the individual effects of metals and temperature on energy metabolism in aquatic organisms, our knowledge of their interactive effects remains deficient (Huegens et al. 2001; Sokolova and Lannig 2008). To date, noteworthy studies on temperature-trace metals interactions on energy metabolism exist for oysters in which a considerable body of knowledge on Cd-temperature interactions has accumulated (Sokolova 2004; Cherkasov et al. 2006a and b; Sokolova and Lannig 2008). There is, however, a clear paucity of information on Cu-temperature interactions in spite of the fact that aquatic organisms including fish are highly sensitive to Cu (Meyer et al. 2007). Notwithstanding its high toxicity in aquatic organisms when in excess, Cu is required for the survival and normal physiology of all organisms (Linder 1991) as a component of more than 30 enzyme systems with crucial biological functions. Among these functions, the role of Cu as a prosthetic group for COX is arguably the most important one because it allows Cu to participate in the all-important aerobic energy metabolism. Additionally, as a component of SOD1, Cu participates in regulating ROS, inevitable by-products of aerobic cellular respiration (Boveris et al. 1972; Halliwell and Gutteridge 1999) with major physiological and pathophysiological effects. These biological roles of Cu are derived from its redox properties and coordination chemistry that allow Cu to act as a catalytic cofactor in proteins (Pena et al. 1999).

Paradoxically, however, the same redox properties make Cu highly toxic when in excess by promoting oxidative stress (Pena et al. 1999) with impairment of cellular energy balance being a primary toxic effect. Specifically, the mitochondria, that are responsible for generating > 90% of the cellular energy requirement via OXPHOS, appear to not only be sites of accumulation but also targets for the toxic action of Cu in a wide range of animal species (Strubelt et al. 1996; Beleyaeva et al. 2004; Krumschnabel et al. 2005; Kamunde and MacPhail 2008; Garceau et al. 2010). The importance of mitochondria in Cu toxicity is further underscored by the fact that Wilson's disease (humans) and canine Cu toxicosis (Bedlington terriers), both genetic disorders characterized by excessive Cu accumulation in the liver, are characterized by mitochondrial dysfunction (Sokol et al. 1994).

In contrast to Cu, which typically presents as a localized chemical stressor in contaminated water bodies, temperature is a pervasive stress factor that typically confounds effects of other stressors due to its overarching effect on biological processes. Indeed, understanding the impact of thermal stress in aquatic systems is becoming increasingly recognized and necessary because in addition to the seasonal and diurnal temperature fluctuations, global climate change is no longer a mere debate but a scientifically defensible phenomenon that is projected to elicit an increase in both the average conditions and frequency of temperature extremes (IPCC, 2007). Temperature fluctuations pose the greatest threat to ectotherms such as fish because their body temperatures, and consequently rates of biological reactions, conform to environmental temperature. Because disturbance in cellular energy homeostasis arguably presents the greatest threat to the fitness, performance and survival of organisms, the need for studies to understand how the principal cellular energy generating units, the mitochondria, respond to thermal stress cannot be overemphasized. In this regard, mitochondrial function has been shown to be highly influenced

by temperature in ectotherms (Abele et al. 2002; Gurderley 2011; Schulte et al. 2011). However, the research carried out to-date on temperature effects in fish remains un-exhaustive, with the large number of species in this class of vertebrates continuing to make it difficult to draw general conclusions.

The integration of effects of multiple stressors requires convergence on a common denominator (Portner 2012). Although the mitochondria (energy metabolism) appear to be a point of convergence for the effects of temperature and Cu ideal for investigating the interactive effects, there is limited information on interactions of these stressors on mitochondrial bioenergetics and fundamental issues including how Cu stress modifies the stereotypical mitochondrial thermal performance curves remain unknown. Moreover, studies on the effects of metals on energy homeostasis in aquatic ectotherms have not generated a unifying theme, an indication that more targeted research is required. The purpose of the present study was therefore to elucidate how Cu stress and temperature moderate each other's effects on mitochondrial function in rainbow trout. I hypothesized that when thermal stress is superimposed on Cu stress, similar or overlapping responses are induced exacerbating mitochondrial impairment.

3.3. Materials and Methods

3.3.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) purchased from Ocean Trout Farm Inc., Brookvale, PE, were held in a 250-l tank with flow-through aerated well water at the Atlantic Veterinary College Aquatic Animal Facility. Fish were fed ad libitum on alternate days with commercial trout chow (Corey Feed Mills, Fredericton, NB) containing, as specified by the manufacturer: crude protein 50% (minimum), crude fat 20% (minimum), crude fiber 1.4% (maximum), calcium 1.7% (17

mg/g actual), phosphorus 1% (actual), sodium 0.6% (actual), vitamin A 2500 IU kg^{-1} (minimum), vitamin D3 2400 IU kg^{-1} (minimum), and vitamin E 200 IU kg^{-1} (minimum). Fish body weight ranged from 120 to 200 g during the course of the experiment. All experimental procedures that fish were subjected to were approved by the University of Prince Edward Island Animal Care Committee in accordance with the Canadian Council on Animal Care (protocol number 11034).

3.3.2. Mitochondrial respiration

Fish were sacrificed by a blow to the head, livers dissected out and mitochondria isolated according to the method detailed in Adiele et al. (2010). Briefly, the livers were rinsed with MIB (composition same as used in chapter 2) and blotted dry. After weighing, the livers were diced into small pieces and homogenized on ice in 3 volumes of MIB using a Potter-Elvehjem homogenizer (Cole Parmer, Anjou, QC). Three passes of a loosely fitting Teflon pestle mounted on a hand-held drill (MAS 2BB, Mastercraft Canada, Toronto) running at 200 rpm provided optimal homogenization. The resulting homogenates were centrifuged at 800 *g*, 4 ^{o}C for 15 min and the supernatants were transferred into fresh centrifuge tubes for a second centrifugation at 13,000 *g*, 4 ^{o}C for 10 min to get mitochondrial pellets. The pellets were washed twice by re-suspending in MIB with centrifugation at 11,000 *g*, 4 ^{o}C for 10 min. The final mitochondrial pellets were weighed and re-suspended in 3 volumes of EGTA-free MRB (composition same as used in Chapter 2) and used for the respiratory experiments. Initially, protein concentrations of mitochondrial suspensions were measured spectrophotometrically (Spectramax Plus 384, Molecular Device, Sunnyvale, CA) by the Bradford (1976) method. Complex II-driven mitochondrial respiration rates were then measured with Clark-type oxygen electrodes (Qubit Systems, Kingston, ON) housed in 4-ml cuvettes. Prior to all the measurements of respiration, a

2-point calibration of the electrodes at 0 and 100% oxygen saturation was done using oxygen depleted (achieved by bubbling N_2) and air-saturated Milli-Q (Millipore, Bedford, MA) water at ambient atmospheric pressure (734-759 mmHg) measured with a traceable digital barometer (Fisher Scientific, Nepean, ON). The cuvettes were initially loaded with 1.45 ml of MRB and continuously stirred for homogenous oxygen distribution. Thereafter, 100 µl of mitochondrial suspensions containing 2-3 mg protein were introduced into the cuvettes followed by 0.5 µM rotenone (CI inhibitor) and 5 mM succinate (CII substrate) using Hamilton syringes fitted with long (6.35 cm) needles. Addition of 200 nmoles of ADP produced state 3 respiration that eventually transitioned to state 4 respiration upon depletion of the ADP. Lastly, oligomycin (2.5 µg ml^{-1}), an inhibitor of CV, was added to measure state 4_{ol}, an estimate of proton leak across the IMM (Brand *et al*., 1994). All the respiration rates were recorded with LabPro® data acquisition software (Qubit Systems) and normalized to mitochondrial protein.

3.3.3. Effect of temperature and Cu on mitochondrial respiration

Mitochondrial respiration was measured at three temperatures: 5 ± 1.0 (low), 11 ± 1.0 (control) and 25 ± 1.0 (high) ^{o}C regulated using a circulating water-bath (Haake, Karlsruhe, Germany). This temperature range falls between the critical thermal minima (0-2 ^{o}C) and maxima (28-29 ^{o}C) for rainbow trout (Finstad et al. 1988; Rodgers and Griffiths 1983; Currie et al. 1998). All the oxygen consumption measurements were completed within 4 h well within the 12 h viability time of isolated rainbow trout liver mitochondria (Adiele et al. 2010). The effects of Cu at each temperature were investigated using six nominal concentrations (0, 0.05, 0.1, 0.5, 1.0 and 2.5 mM) of Cu [as $CuSO_4$•$5H_2O$, Sigma-Aldrich Oakville, ON]. This dose range is wide and includes low and high concentrations that cannot be encountered in cells under real exposure situations but nonetheless permits me to investigate mechanisms of Cu-induced mitochondrial

dysfunction. The dose selection also took into account the brief exposure periods used and the fact that the respiration buffer contains BSA which binds Cu reducing the bioavailable (effective) concentration. Moreover, the only available study regarding the effect of Cu on mitochondria from an aquatic species (Collins et al. 2010) used 1-20 mM Cu dose range wherein 1 mM Cu caused about 20% inhibition of state 3 respiration. Note that although the respiration rates varied substantially across the three temperatures, the contact time of the mitochondria and Cu was kept the same for valid comparison of the results. Specifically, all the state 3, 4 and 4_{ol} rates of respiration were measured after 2.5 ± 0.5, 4.5 ± 0.5 and 7 ± 0.5 min of exposure to Cu, respectively.

3.3.4. Q_{10} and activation energy (E_a) calculations

Q_{10} values for state 3, 4 and 4_{ol} respiration rates were calculated for the temperature ranges 5-11, 11-25 and 5-25 °C using the equation:

$$Q_{10} = (R2/R1)^{[10/(T2-T1)]} \qquad \text{(Eq. 1)}$$

where $R2$ and $R1$ are the mitochondria oxygen consumption rates at the respective temperatures $T2$ and $T1$ (where T2 > T1).

The activation energies (E_a) for state 3, 4 and 4_{ol} respiration rates were calculated for the temperature range 5-25 ^{o}C based on the Arrhenius equation:

$$E_a = (R \times \ln(k2/k1) \times (T1 \times T2))/(T2-T1) \qquad \text{(Eq. 2)}$$

where E_a is activation energy (J mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}), k1 and k2 are mitochondrial respiration rates at the respective temperatures, T1 and T2 in Kelvin (where T2 > T1).

3.3.5. Complex II (succinate:ubiquinone oxidoreductase) activity

Mitochondria were isolated as described above, re-suspended in MRB and 95 µl aliquots added into 1.5 ml centrifuge tubes. Subsequently 5 µl of Cu stock solution (as $CuSO_4 \bullet 5H_2O$) was added to deliver desired concentrations (0-160 µM) in a final volume of 100 µl and mixed gently. Each Cu exposure was done in duplicate for 5 mitochondrial preparations from 5 different fish (i.e., n = 5). For control mitochondria, 5 µl of MRB was added instead of the Cu stock solution. The mitochondria were then incubated at room temperature for 30 or 60 min under continuous mixing. After incubation the mitochondria were washed (to stop continued exposure to Cu after the timed incubation) twice by re-suspending in 500 µl of MIB, mixing by gentle vortexing and centrifuging at 10,000 *g* for 5 min at 4 ^{o}C. The resulting pellet was re-suspended in 100 µl MRB and used for measuring CII activity according to the method of Spinazzi et al. (2012) with a few modifications. In this redox assay, succinate is the donor and DCPIP is the acceptor of electrons. First, the mitochondrial suspensions were diluted to 6 mg/ml in hypotonic buffer and sonicated to disrupt the IMM. Thereafter, 60 µg of mitochondrial protein (10 µl) was added to 240 µl of assay buffer (pH 7.3) containing 25 mM potassium phosphate, 18 mM sodium succinate, 0.1 mM DCPIP, 2.5 mM KCN, 2.5 µg/ml antimycin A, and 10 µM rotenone. Absorbance at 600 nm was read (Spectramax Plus 384) for 3 min to establish a baseline. The reaction was then initiated by addition of 10 µl of 1.625 mM coenzyme Q_1 and the decrease in absorbance as a result of reduction of DCPIP was recorded every 15 s for 5 min. CII activity was calculated using the DCPIP molar extinction coefficient of 19.1 mM^{-1} cm^{-1}.

3.3.6. Data analysis

The data were checked for normality of distribution and homogeneity of variances and submitted to two-way ANOVA (Statistica version 5.1, Statsoft, Inc., Tulsa, OK) with "temperature" and "Cu dose" (respiration data) and "duration of incubation" and "Cu dose" (enzyme activity data) as the independent variables. The Q_{10} and E_a data were analyzed with one-way and two-way ANOVA with "Cu dose" (Q_{10}) and "respiration state" and "Cu dose" (E_a) as the independent variables, respectively. Differences among mean values were delineated using Tukey's post hoc test at $p < 0.05$. The Cu doses that caused 50% inhibition (ED_{50}) of state 3 respiration and CII activity were calculated using Sigma Plot 10 (Systat Software Inc., San Jose, CA).

3.4. Results

3.4.1. Effect of temperature and Cu on mitochondrial respiration

The state 3 mitochondrial respiration rate depended on the temperature and increased almost 3-fold from 11.4 to 31.7 nmol O_2 mg $prot^{-1}$ min^{-1} over the 5 to 25 ^oC temperature range (Fig. 3.1A). Copper exhibited a threshold dose between 0.05 and 0.1mM, with higher doses markedly inhibiting state 3 respiration with terminal reductions in oxygen consumption (in the 2.5 mM dose) of 50, 73 and 98% at 5, 11, and 25 ^oC, respectively. High temperature exacerbated the inhibitory effect of Cu on mitochondrial maximal respiration as evident from the state 3 thermal performance curves (Fig. 3.1B) and ED_{50} values of 480, 272, and 98 µM Cu at 5, 11 and 25 ^oC, respectively. Correspondingly, the state 3 respiration Q_{10} effect values for the 5 to 25 ^oC range (Fig. 3.1C) decreased dose-dependently from a control value of 1.7 to 0.25 at 2.5 mM Cu. Generally, the Q_{10} values were higher for the 5-11 ^oC range (data not shown).

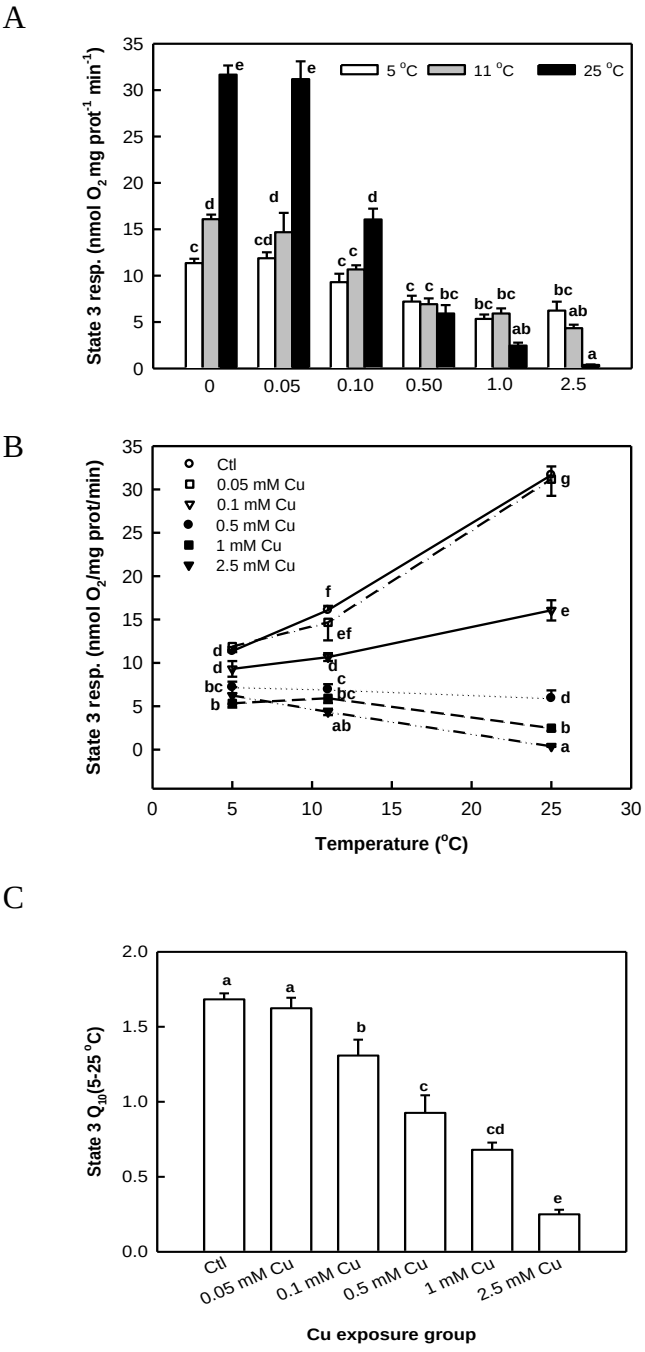


Figure 3.1. Interactions of temperature and Cu stress on rainbow trout liver mitochondrial bioenergetics. A: maximal (state 3) respiration; B: state 3 thermal performance curves; C: state 3 Q_{10}. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (ANOVA with Tukey's HSD, $p < 0.05$).

The state 4 respiration rate in the absence of Cu was appreciably temperature-dependent and increased by more than 4 times from 3.8 (nmol O_2 mg $prot^{-1}$ min^{-1}) at 5 ^oC to 16.7 at 25 ^oC (Fig. 3.2A). Copper exposure imposed a biphasic response on state 4 respiration with stimulation by doses ≤0.1 mM and inhibition by higher doses (Fig. 3.2A). This response was reflected in the thermal performance curves in which low Cu doses increased, whereas high Cu doses reduced, the sensitivity of resting respiration to temperature (Fig. 3.2B). Notwithstanding the biphasic response, the overall effect of Cu was a dose-dependent reduction in state 4 Q_{10} values from 2.1 (control) to 0.27 at 2.5 mM Cu (Fig. 3.2C). The related state 4_{ol} respiration (Fig. 3.3A-C) clearly increased with temperature in the absence of Cu (Fig. 3.3A). Copper exposure elicited a biphasic response at 5 and 11 ^oC wherein low doses (≤0.1 mM) exacerbated and high doses inhibited the proton leak. Contrastingly, a monotonic decrease in proton leak with increasing Cu dose was observed at 25 ^oC (Fig. 3.3A). Although the thermal performance curves (Fig. 3.3B) indicate that low Cu doses increased the thermal sensitivity of proton leak, overall for the 5-25 ^oC temperature range the Q_{10} values decreased dose-dependently from 1.9 to 0.35 (Fig. 3.3C).

The mitochondrial coupling efficiency (RCR), calculated according to Estabrook (1967), was variably influenced by Cu exposure and temperature (Fig. 3.4A). In the absence of Cu exposure, the RCR decreased temperature-dependently from about 3 at 5 ^oC to 2 at 25 ^oC. Copper exposure reduced the RCR at 5 and 11 ^oC but had no effect at 25 ^oC except that the 0.5 mM dose significantly increased the RCR from 2 (at control Cu dose) to 3.9. Although a comparable response to temperature and Cu exposure was observed with regards to RCR_{ol}, only the 0.5 mM Cu dose at 25 ^oC resulted in a clearly elevated RCR_{ol} value (Fig. 3.4B).

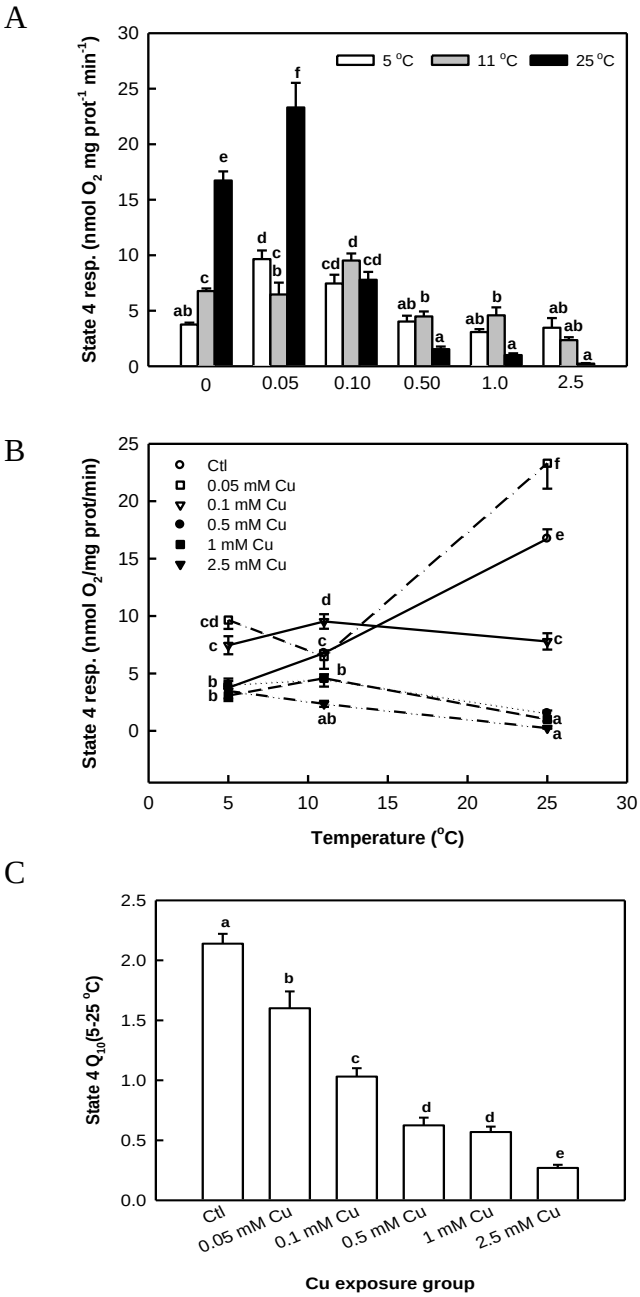


Figure 3.2. Interactions of temperature and Cu stress on rainbow trout liver mitochondrial bioenergetics. A: resting/state 4 respiration; B: state 4 thermal performance curves; C: state 4 Q_{10}. The Q_{10} values are for 5-25 °C temperature range. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (ANOVA with Tukey's HSD, $p < 0.05$).

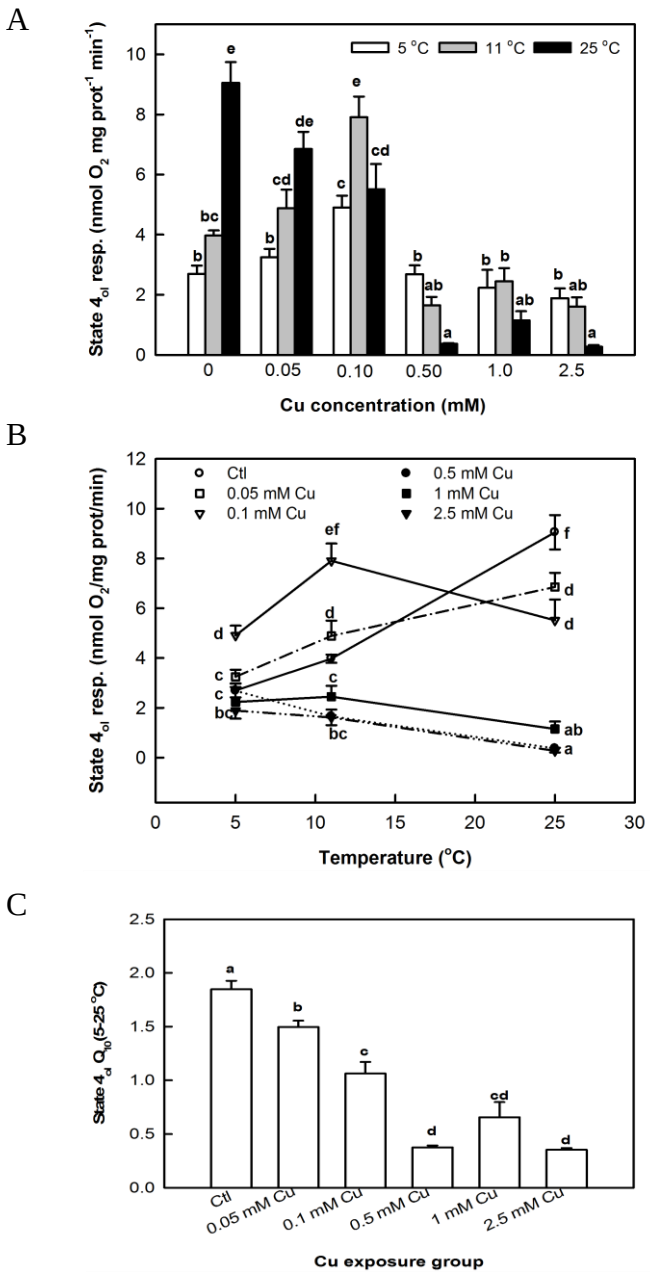


Figure 3.3: Interactions of temperature and Cu stress on rainbow trout liver mitochondrial bioenergetics. A: proton leak (state 4_{ol} respiration); B: proton leak thermal performance curves; C: proton leak Q_{10}. The Q_{10} values are for 5 to 25 ^{o}C temperature range. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (ANOVA with Tukey's HSD, $p < 0.05$).

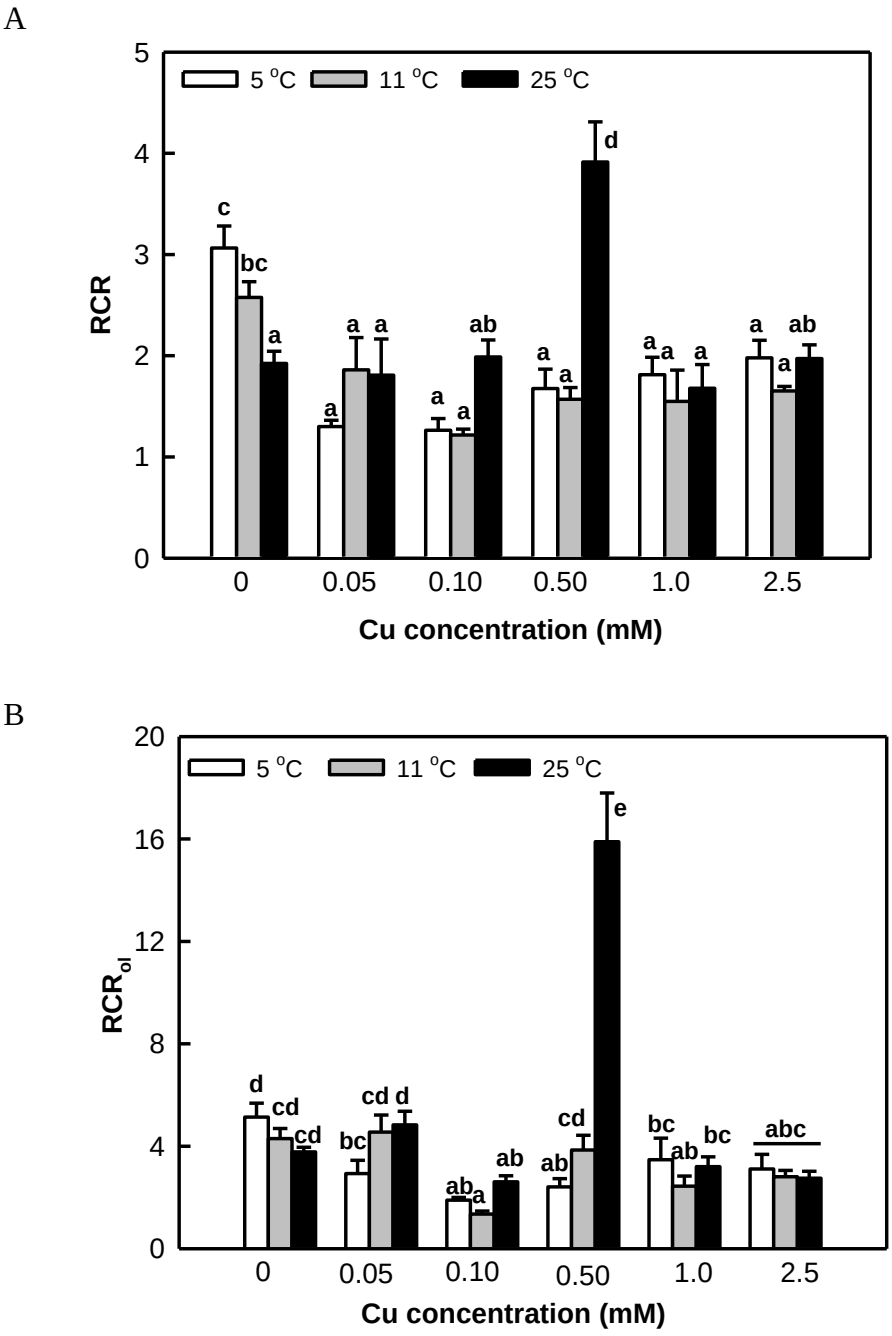


Figure 3.4: Effect of temperature and Cu stress on rainbow trout liver mitochondrial coupling efficiency. A: RCR; B: RCR_{ol}. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (ANOVA with Tukey's HSD, $p < 0.05$).

Activation energies for all respiration states were higher for the 5-11 compared to the 5-25 and 11-25 ^{o}C temperature ranges (data not shown). For the whole thermal range (5-25 ^{o}C) Cu exposure drastically reduced the E_as for all the mitochondrial respiration states (Fig. 3.5). Specifically, the state 3, 4 and 4_{ol} E_as decreased from 35 to -97, 51 to -91 and 42 to -71 kJ mol^{-1}, respectively, following exposure to the highest Cu dose (2.5 mM). Overall, the E_as for the three respiration states became negative on exposure to Cu doses > 0.1 mM.

3.4.2. Mitochondrial complex II (CII) activity

The effect of Cu on CII activity assessed with 0-160 μM Cu (Fig. 3.6A) revealed that similar to the effect of Cu on succinate-driven mitochondrial respiration, low Cu concentrations stimulated whereas high Cu concentration inhibited the activity of this enzyme following 30 min incubation. Incubation of the mitochondria with Cu for 60 min elicited greater inhibition of CII activity without a stimulatory effect at low doses. The computed Cu doses required to inhibit CII activity by 50% (ED_{50}) were, respectively, 74.2 and 54.5 μM for the 30 and 60 min incubations (Fig. 3.6B).

3.5. Discussion

Concurrent or sequential exposure of biological systems to multiple stressors induces complex (additive, antagonistic or synergistic) interactive effects that are difficult to model and predict from single stressor effects (Folt et al. 1999; Christensen et al. 2006; Altshuler et al. 2011). In the present study I assessed how two key environmental stressors –temperature and Cu– that commonly co-occur in aquatic systems interact on mitochondrial bioenergetics to shed light on their joint action. Moreover, the effects of Cu and temperature as single stressors in fish mitochondria have not been exhaustively investigated.

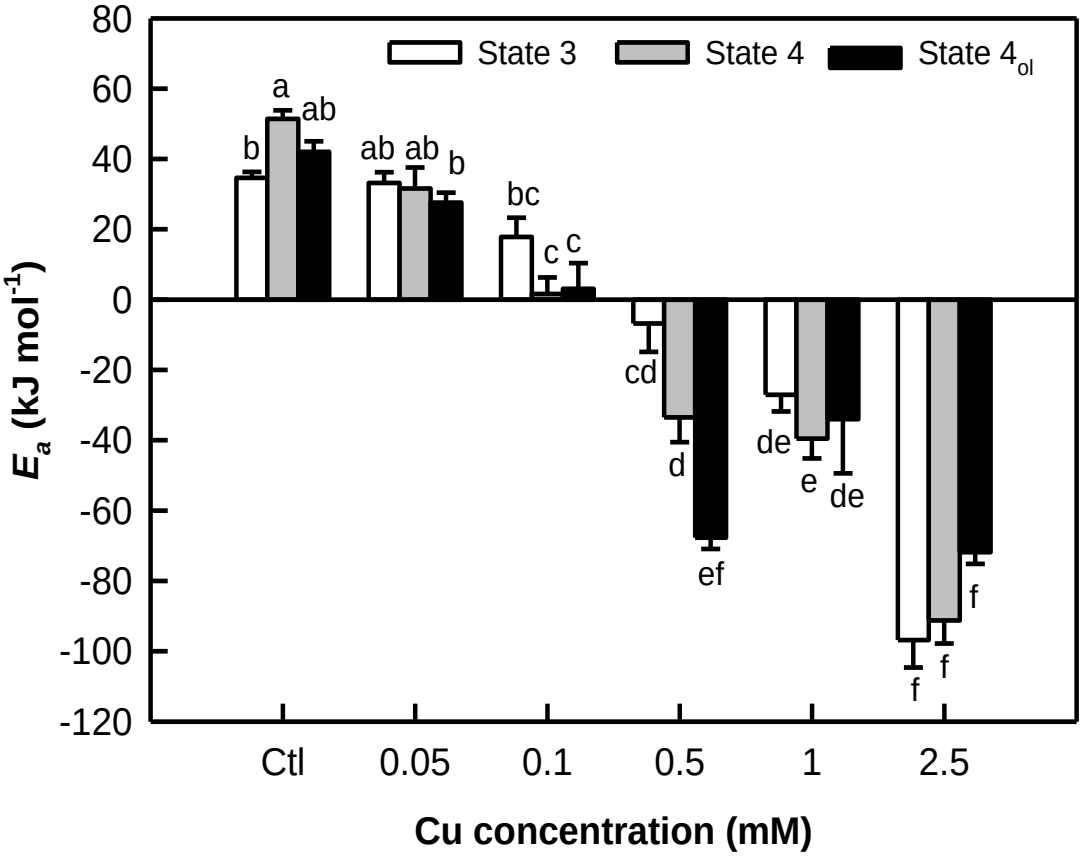
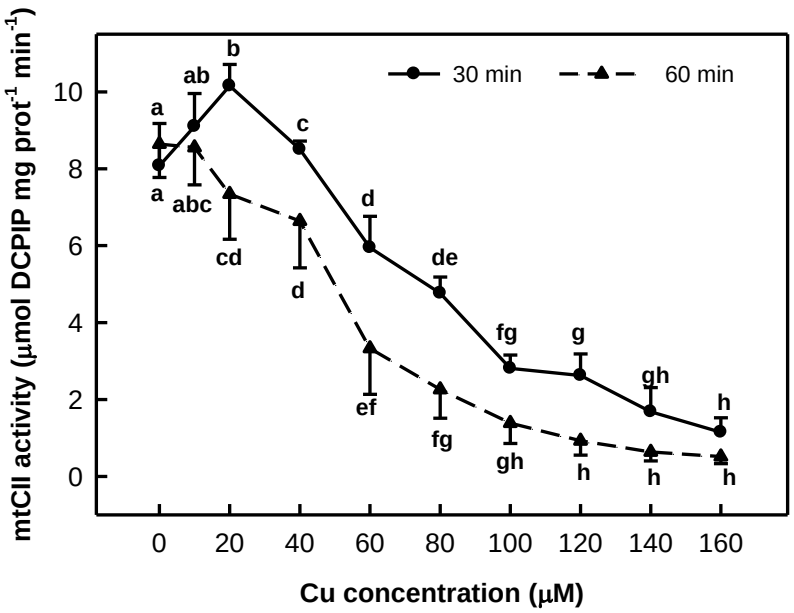


Figure 3.5: Effect of temperature and Cu stress on rainbow trout liver mitochondria state 3, 4 and 4_{ol} activation energies (E_a). Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (ANOVA with Tukey's HSD, $p < 0.05$).

A



B

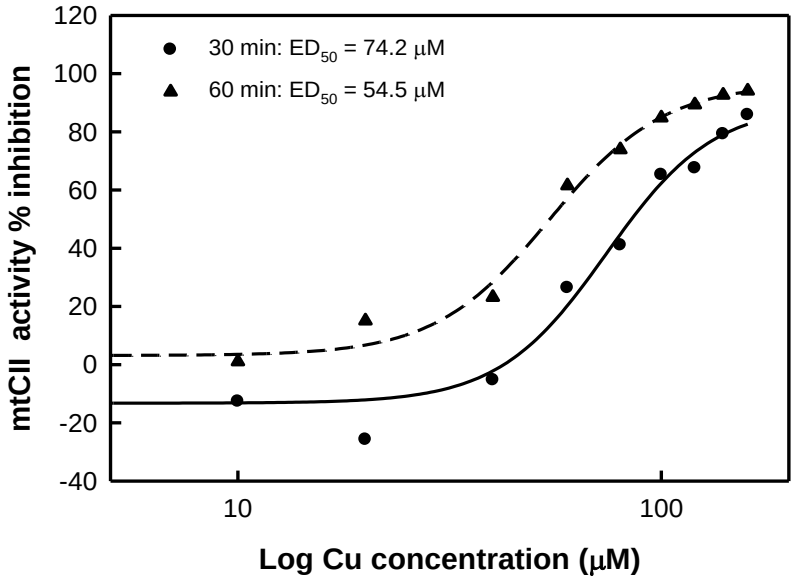


Figure 3.6: Effect of Cu on rainbow trout liver mitochondria CII activity. A: complex II activity following 30 (circle) and 60 (triangle) incubation with Cu at room temperature. Data are means ± SEM (n = 5). Points with different letters are significantly different from each other (two-way ANOVA with Tukey's HSD, $p < 0.05$). B: CII Cu ED_{50}.

The results demonstrate that the effect of Cu as a single stressor depends on the dose and energetic status of the mitochondria; low Cu (<0.1 mM) doses stimulated resting respiration without affecting phosphorylating respiration while higher (>0.1mM) doses inhibited both respiration states. Copper-induced inhibition of maximal respiration/ETS activity has previously been observed in the eastern oyster *Crassostrea virginica* (Collins et al. 2010) and goldfish, *Carassius auratus* (Garceau et al. 2010) mitochondria as well as in rat brain homogenate (Heron et al. 2001) and may be attributed to decreased substrate oxidation or inhibition of phosphorylation. In the present study, the inhibition of CII activity (Fig. 3.6) confirms involvement of the substrate oxidation subsystem in Cu-induced mitochondrial dysfunction.

The stimulation of resting and proton leak (state 4_{ol}) respiration by low doses of Cu observed in the present study is consistent with earlier studies in rats (Saris and Skulskii 1991; Belyaeva et al. 2004, 2011) and fish (Manzl et al. 2003). Mitochondrial proton leak is believed to be mediated by the ANT, UCPs and other IMM proteins (Parker et al. 2008; Jastroch et al. 2010). Conceivably, low Cu doses stimulated these mechanisms. Additionally, Cu exposure increases mitochondrial generation of ROS which not only activate mechanisms of proton leak (Echtay et al. 2002; Jastroch et al. 2010) but also induce MPTP and oxidize mitochondrial membrane lipids thus increasing membrane leakiness to protons. Another possible explanation of the Cu-induced proton leak is electrogenic transport of Cu ions into the mitochondrial matrix. Incidentally, electrogenic Cu transport through a Na^+/Ca^{2+} exchanger has been observed in the American lobster (*Homarus americanus*) hepatopancreatic mitochondria (Chavez-Cocker et al. 2002). Regardless of the cause, increased proton leak can have two disparate effects during Cu exposure. First, because H^+ leak reduces MMP which minimizes generation of ROS (Abele et al. 2002), it may attenuate ROS-mediated toxic effects of Cu. In this regard Cu exposure dissipated

the membrane potential in mitochondria isolated from rat liver (Belyaeva et al. 2004) and anterior gills of hyperosmoregulating estuarine crab, *Neohelice granulate* (Lauer et al. 2012) as well as in rat hepatocytes (Pourahmad and O'Brien 2000) and cortical neuronal cells (Sheline and Choi 2004). Second, reduced ATP generation and increased energy requirement for mitochondrial maintenance due to high proton leak (Wallace and Starkov 2000; Sokolova 2004, Cherkasov et al. 2006a; Sokolova and Lannig 2008) can aggravate Cu toxicity because cellular handling of metals requires energy (Kumar and Couture 2003).

Elevated temperature increased the maximal, resting and leak respirations in line with the theme that ectothermic mitochondrial function is highly influenced by temperature (Abele et al. 2002; Guderley 2011; Schulte et al. 2011). In the absence of Cu, maximal respiration had a Q_{10} value of 1.7 which is within the range of 1.3 to 3.4 measured in both ecto- and endothermic mitochondria (Dufour et al. 1996; Portner et al. 1999a; Kraffe et al. 2007). Interestingly, although it has been claimed that resting mitochondrial respiration has low sensitivity to temperature in ectotherms (Pye 1973; Almeida et al. 1994), I observed considerable thermal sensitivity of state 4 and 4_{ol} with Q_{10} values of approximately 2. These findings, including earlier studies in Antarctic fish and bivalves that reported Q_{10} values for proton leak well above 2 (Hardewig et al. 1999a; Portner et al. 1999a; Mark et al. 2012), support the theme that proton leak is driven by active processes. Note that passive diffusive processes are characterized by Q_{10} values close to 1 (Guderley and St-Pierre 2002). Overall, due to the preferential thermal sensitivity of resting relative to maximal respiration, elevated temperature reduced the mitochondrial coupling efficiency. Surprisingly, previous studies reported that coupling of ectothermic mitochondria is temperature-independent (Pye 1973; Moyes et al. 1988; Blier and Guderley 1993; Almeida et al. 1994; Guderley and Johnston 1996; Mark et al. 2012) leading to

the generalization that temperature-induced uncoupling is a trait of mammalian (endothermic) mitochondria (Luvisetto et al. 1992) rarely observed in ectotherms (Hardewig et al. 1999a). Therefore studies are required to understand why some ectothermic mitochondria possess characteristics typical of mammalian mitochondria.

The few studies on temperature-metals interactions on mitochondrial bioenergetics (Sokolova 2004, Cherkasov et al. 2006a and b; Lannig et al. 2006; Ivanina et al. 2008) show that Cd impairs the ability of the eastern oyster mitochondria to respond to temperature whereas elevated temperature increases the sensitivity of mitochondria to Cd. In the present study, low Cu levels combined with high temperature increased mitochondrial inefficiency by promoting proton leak while high temperature exacerbated the inhibitory effects of Cu on mitochondrial respiration as reflected by lower state 3 respiration Cu ED_{50} at 25 ^{o}C. The overall interactive effect, reflected in the reduced Q_{10} and E_a values, suggests that mitochondria impacted by Cu have reduced capacity to regulate OXPHOS during thermal stress. Thus while the reduced E_a would conceivably facilitate metabolic flux, elevated Cu appears to impose severe thermal constraints on the biochemical processes involved in OXPHOS leading to marked reduction in mitochondrial aerobic scope. Reduced E_a for mitochondrial respiration and specific mitochondrial enzymes, notably with some negative E_a values as observed in the present study, has previously been reported with regards to Cd-temperature interactions on the eastern oyster mitochondria (Ivanina et al. 2008) suggesting that Cu and Cd affect mitochondrial temperature sensitivity in diverse animal groups in a similar manner. Overall, relative to single stressor effects, the combined action of elevated temperature and Cu caused greater impairment of mitochondrial aerobic capacity reaffirming the notion that sensitivity of aquatic organisms to metals increases with temperature (Heugens et al. 2001; Sokolova and Lannig 2008; Altshuler et al. 2011).

The unambiguously increased coupling observed at 25 ^{o}C with mid-range (0.5 mM) Cu dose (Fig. 3.4) was surprising and leads me to hypothesize that under some conditions, interactions between multiple stressor may result in beneficial or protective effects. Although in the present study the observed increased coupling may simply be a computational artifact reflecting the temperature-Cu combination at which state 3 respiration was minimally inhibited relative to the state 4 stimulation, Onukwufor et al. (2014) recently demonstrated a protective effect of low doses of Cd on HRO-induced mitochondrial proton leak. Likewise, exposure of mantle cells isolated from hard clams (*Mercenaria mercenaria)* to acute hypercapnia reduced intracellular pH which in turn attenuated Cd-and Cu-induced ROS production (Ivanina et al. 2013). It is foreseeable that as the number and range of studies on interactive effects of multiple stressors increase, more of these unexpected potentially beneficial responses will be unearthed.

The Cu-induced impairment of OXPHOS observed in the present study was due, at least in part, to inhibition of CII. Because CII participates both in the TCA and electron transport, its inhibition results in reduced production of high energy molecules (NADH and $FADH_2$) and delivery of fewer electrons to the Q pool of the ETS. Consequently, fewer protons are pumped to the IMS by the distal ETS complexes (III and IV) leading to reduction of the pmf which drives ATP synthesis. Although not investigated here, inhibition of CII by Cu may involve oxidative damage to components of the enzyme because Cu exposure is known to stimulate ROS formation in rainbow trout hepatocytes (Manzl et al. 2003; Krumschnabel et al. 2005). The present results and recent finding that ETS complexes (e.g., CII) which have Fe-S centers are sensitive to inhibition by Cd (Adiele et al. 2012) suggest that, similar to Cd, Cu impairs CII Fe-S centers. Interestingly, the Cu ED_{50} calculated for CII activity in the present study was within the range of Cu concentrations accumulated by fish in subcellular compartments following exposure

to environmentally relevant Cu concentrations (Garceau et al., 2010). Thus CII appears to be an environmentally relevant target for Cu. Importantly, the greater impairment of mitochondrial function observed when Cu exposure was performed at elevated temperature can partly be explained by the fact that effects of inhibition of CII added to those of temperature (e.g., increase in proton leak) to exacerbate mitochondrial dysfunction.

In summary, the present study shows that while low Cu doses impair mitochondrial bioenergetics by stimulating proton leak, high Cu doses primarily inhibit maximal respiration. Copper-induced mitochondrial impairment results from direct inhibition of CII enzyme activity and stimulation of state 4_{ol} suggesting that both substrate oxidation and proton leak subsystems of the mitochondria are targets of deleterious effects of Cu. High temperature promotes mitochondrial uncoupling by stimulating proton leak and sensitizes rainbow trout mitochondria to Cu toxicity while Cu reduces thermal sensitivity of OXPHOS. Because mitochondrial aerobic capacity reflects the whole organism aerobic scope, these results suggest that global energy homeostasis in fish would be disturbed at lower Cu levels in presence of elevated temperature.

CHAPTER 4

ALTERATIONS IN MITOCHONDRIAL ELECTRON TRANSPORT SYSTEM ACTIVITY IN RESPONSE TO WARM ACCLIMATION, HYPOXIA-REOXYGENATION AND COPPER IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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4.1. Abstract

Fish expend significant amounts of energy to handle the numerous potentially stressful biotic and abiotic factors that they commonly encounter in aquatic environments. This universal requirement for energy singularizes mitochondria, the primary cellular energy transformers, as fundamental drivers of responses to environmental change. My study probed the interacting effects of thermal stress, HRO and Cu exposure in rainbow trout to test the prediction that they act jointly to impair mitochondrial function. Rainbow trout were acclimated to 11 (controls) or 20 °C for 2 months. Liver mitochondria were then isolated and their responses *in vitro* to Cu (0-20 μM) without and with HRO were assessed. Sequential inhibition and activation of mitochondrial ETS enzyme complexes permitted the measurement of respiratory activities supported by complex 1-IV (CI-IV) in one run. The results showed that warm acclimation reduced fish and liver weights but increased mitochondrial protein indicating impairment of energy metabolism, increased synthesis of defense proteins and/or reduced liver water content. Whereas acute rise (11→20 ^{o}C) in temperature increased mitochondrial oxidation rates supported by CI-IV, warm acclimation reduced the maximal and increased the basal respiration leading to global uncoupling of OXPHOS. HRO profoundly inhibited both maximal and basal respiration rates supported by CI-IV, reduced RCR for all except CII and lowered CI:CII respiration ratio, an indication of decreased OXPHOS efficiency. The effects of Cu were less pronounced but more variable and included inhibition of CII-IV maximal respiration rates and stimulation of both CI and CIII basal respiration rates. Surprisingly, only CII and CIII indices exhibited significant 3-way interactions whereas 2-way interactions of acclimation either with Cu or HRO were portrayed mostly by CIV, and those of HRO and Cu were most common in CI and II respiratory indices. My study suggests that warm acclimation blunts sensitivity of the ETS

to temperature rise and that HRO and warm acclimation impose mitochondrial changes that sensitize the ETS to Cu. Overall, my study highlights the significance of the ETS in mitochondrial bioenergetic dysfunction caused by thermal stress, HRO and Cu exposure.

4.2. Introduction

Aquatic systems particularly in temperate zones may experience wide changes in daily and annual mean temperatures (Bridges 1988), the severity of which is expected to increase due to global climate change (Ficke et al. 2007; Doney et al. 2012). These changes greatly influence fish physiology both in the short and long-term because being ectothermic, fish body temperatures conform to those of their environment (Hochachka and Somero 2002). Specifically, changes in fish internal body temperatures driven by environmental temperature alter the kinetic energy of molecules thereby modifying rates of diffusion, molecular interactions and membrane properties, all of which govern cellular biochemical reactions (Guderley and St-Pierre 2002). In addition to conforming to environmental temperature, fish also adjust to persistent temperature change through physiological plasticity (acclimation), biochemical adaptation, or by moving to more suitable thermal habitats (Kraffe et al. 2007). While many biological processes mediate acclimation to temperature change, the mitochondria, by virtue of being the primary cellular energy converters, are arguably the fundamental drivers of responses to thermal change. For example, when low temperature persists, fish modify mitochondrial structure and function to enhance aerobic metabolism thus compensating for functional and physical limitations imposed by low temperature (Kraffe et al. 2007; Guderley 2004, 2011). Acclimation to temperature change also involves HVA with alterations in the level of saturation and relative proportions of fatty acids in phospholipids to maintain membrane functional integrity (Hazel 1995; Kraffe et al. 2007). Notably, temperature-imposed changes in cardiolipin, a phospholipid localized primarily in the IMM and known to establish interactions with ETS enzyme complexes that are critical for their energy transducing function (Houtkooper and Vaz 2008; Schlame and Ren 2009; Paradies et al. 2014), can preferentially and profoundly impair mitochondrial bioenergetics.

Similar to temperature, environmental hypoxia is a common, naturally occurring phenomenon in many aquatic systems, the prevalence of which is increasing as a result of anthropogenically induced eutrophication (Diaz 2001; Diaz and Breitburg 2009). Fish respond to hypoxia using a suite of physiological, biochemical and molecular responses that either maintain O_2 uptake from the environment or mitigate the deleterious effects of reduced cellular O_2 levels (Hochachka et al. 1996; Richards 2009, 2011). Overall these responses alter metabolic energy production and utilization that permit survival under reduced O_2 levels (Richards 2009). Interestingly, the deleterious effects of hypoxia are not limited to the consequences of reduced cellular O_2 but extend to the phase of oxygen availability (reoxygenation). This second phase is due to a surge in production of ROS, primarily by mitochondria, when O_2 becomes available to cells that were previously deprived of it (Caraceni et al. 1995; Korge et al. 2008).

Clearly, both temperature and hypoxia significantly influence cellular energy metabolism in fish. Importantly, hypoxia and thermal stress appear to be inextricably linked in that low temperature as experienced in aquatic habitats under winter conditions can lead to hypoxic conditions (Guderley and St-Pierre 2002; Ultsch 2006) while high temperature decreases O_2 solubility which can similarly lead to hypoxia. Functionally and evolutionarily, the basis of temperature and low DO levels as interacting stressors in aquatic systems can be depicted by the concept of OCLTT (Portner 2010; Portner and Knust 2007; Portner and Lannig 2009). This concept postulates that at warm and cold temperatures, aerobic metabolism would be compromised due to inequality between mitochondrial O_2 requirement and O_2 delivery or by mitochondrial catalytic constraints.

The interwoven roles of hypoxia and temperature in aquatic systems can potentially be cofounded by chemical pollutants, e.g., metals that are derived from both anthropogenic and

natural sources. Although thermal stress, hypoxia and metals have individually been shown to impact energy metabolism, few studies have attempted to elucidate the mechanisms and consequences of hypoxia-temperature-metals stress interaction on mitochondrial bioenergetics. Specifically, there is to the best of my knowledge, no study that has assessed the effects and interactions of these factors on the respiratory function of the four (CI-IV) mitochondrial electron transporting enzyme complexes simultaneously. Targeting multiple ETS components in one run could reveal their relative contribution to mitochondrial bioenergetic function/dysfunction, and unveil the mechanisms that underlie organismal compensation of stressor-induced deleterious effects that are coupled to energy metabolism. The present study therefore probed how acclimation to warm temperature influences mitochondrial ability to handle acute temperature rise, HRO and Cu stress. Isolated mitochondria were used to gain insights into the mechanisms that might underlie cellular and organismal alterations imposed by these stressors. I hypothesized that (i) warm acclimation would minimize mitochondrial dysfunction associated with acute temperature rise but increase the effects of HRO and Cu exposure, (ii) based on shared mechanisms of action, warm acclimation would abrogate the effects of HRO and Cu on mitochondrial bioenergetics through the phenomenon of cross-tolerance and (iii) the effects of the three stressors on mitochondrial respiratory flux would be similar irrespective of the ETS segment activated. Overall, I sought to identify segments and components of the ETS that are resilient and susceptible to single and combined effects of temperature, Cu and HRO.

4.3. Materials and methods

4.3.1. Ethics

All of the experimental procedures that fish were subjected to were consistent with the Canadian Council on Animal Care guidelines as approved by the University of Prince Edward Island Animal Care Committee.

4.3.2. Experimental animals and warm acclimation

Rainbow trout (*Oncorhynchus mykiss*) were purchased from Ocean Trout Farm Inc., Brookvale, PE. Following acclimatization to laboratory conditions at the Atlantic Veterinary College Aquatic Animal Facility, fish were divided into two groups in 250-l tanks supplied with flow-through aerated well water. One group was maintained under control (cold) temperature (11 °C) and the other was acclimated to warm (20 °C) temperature for 2 months. For acclimation, the water temperature was increased gradually over 10 days and acclimation period denotes time after the desired (20 °C) temperature was achieved. The temperature was monitored twice daily and did not deviate significantly from the desired levels. Note that while a temperature of 11 ^{o}C is considered optimal, 20 °C is below the critical thermal maxima for rainbow trout (Rodgers and Griffiths 1983; Finstad et al. 1988; Currie et al. 1998). Dissolved O_2, CO_2, pH and ammonia levels were monitored throughout the acclimation period and were 9.5-9.62 mg/l, 1 mg/l, 7.3-7.5 and 0.08-0.11 mg/l for the 11 °C tank, and 8.5-8.99 mg/l, 2 mg/l, 7.5-8.02 and 0.06-0.18 mg/l for the 20 °C tank, respectively. Fish were fed daily at 1% body weight with commercial trout chow (Corey Feed Mills, Fredericton, NB) with the ration being adjusted weekly based on the weight of every fish in each tank measured weekly during the acclimation period. Note that this ration did result in increased fish body mass in both groups.

4.3.3. Isolation of liver mitochondria

To isolate liver mitochondria, fish were randomly netted from holding tanks and rapidly killed by a cephalic blow. Fish were weighed and the livers immediately dissected out, weighed, rinsed with ice-cold MIB (composition was same as in chapter 2) and blotted dry. Thereafter mitochondrial isolation was done according to our routine procedure (Sappal et al. 2014a,b). Upon isolation, mitochondria were re-suspended in 3 volumes of MRB (composition was same as in chapter 2). The protein concentrations of the suspensions were measured spectrophotometrically (Spectramax Plus 384, Molecular Device, Sunnyvale, CA) by the Bradford (1976) method before the addition of BSA. For each experimental group, 5 mitochondrial preparations from livers of 5 different fish (n = 5) were made, kept on ice and the respiration experiments were done within 4 h of isolating the mitochondria.

4.3.4. Measurements of mitochondrial respiration under normoxic conditions

Using a sequential ETS inhibition and activation protocol (Fig. 4.1) respiration rates driven by mitochondrial CI, CII, CIII and CIV were measured in one run with Clark-type oxygen electrodes (Qubit systems, Kingston, ON). An initial experiment assessed the effect of acute temperature rise wherein respirations driven by CI-IV for liver mitochondria isolated from fish maintained at 11 ^{o}C were measured at 20 ^{o}C. All of the other measurements were done at the respective acclimation temperatures (i.e., 11 or 20 ^{o}C) and cuvette temperature was maintained with a thermostatically controlled water circulator (Haake, Karlsruhe, Germany). Prior to the respiratory measurements, the oxygen electrodes were calibrated at 0 and 100% air saturation by bubbling N_2 and air to milli-Q water, respectively, at ambient atmospheric pressure (740-760 mmHg) measured by a digital barometer (Fisher Scientific, Nepean, ON).

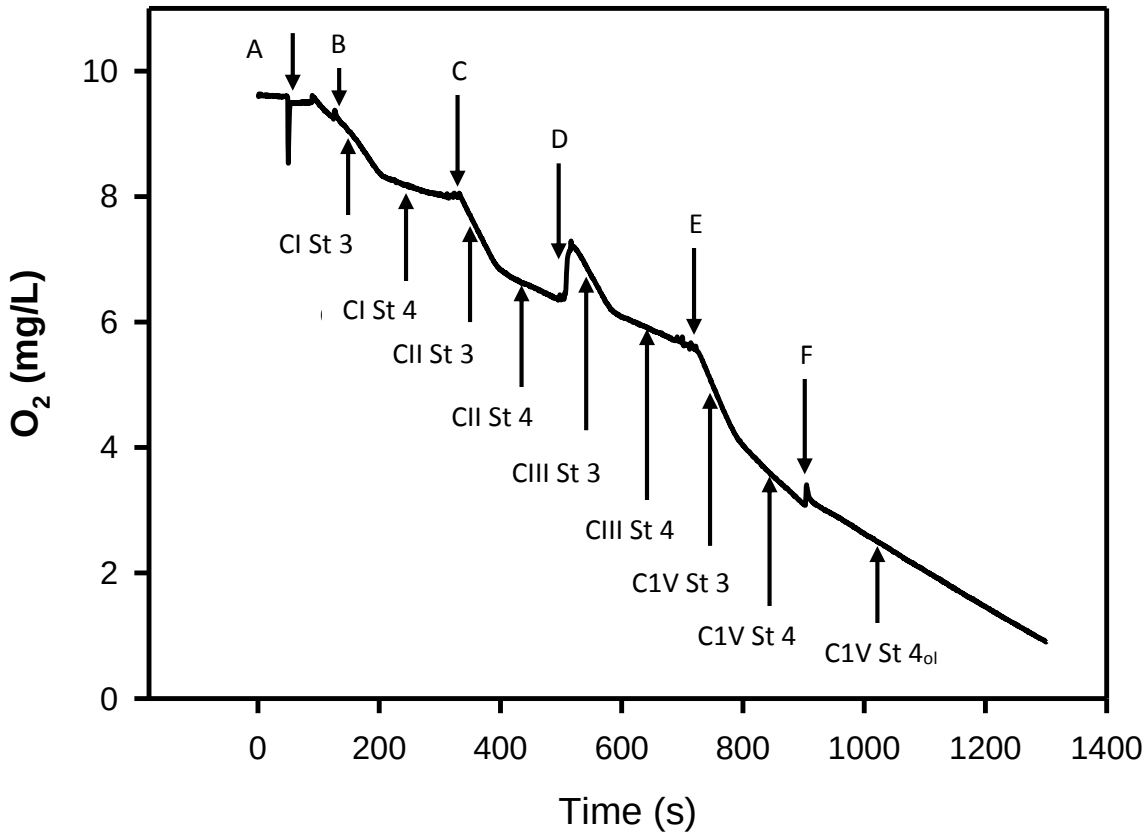

Figure 4.1. Typical polarographic tracing (control, 20 °C) showing sequential inhibition and activation protocol for measuring mitochondrial ETS complex I-IV (CI-IV) respiratory activity in one run. A: addition of mitochondrial suspension; B: addition of ADP after glutamate-malate; C: addition of succinate + ADP after rotenone, CI inhibitor; D: addition of decylubiquinol + ADP after CII inhibitor, malonate; E: addition of ascorbate-TMPD + ADP after CIII inhibitor, antimycin; F: addition of CV inhibitor, oligomycin. Post hypoxia-reoxygenation (HRO) measurements were obtained in a similar manner after exposing the mitochondria to 10 min HRO as described earlier (Onukwufor et al. 2014). Effects of Cu were measured by adding the metal once after CI substrates + ADP without and after HRO. St = state.

Initially, the MRB was equilibrated to experimental temperature (11 or 20 °C) by placing in a water bath at the desired temperature. Subsequently, cuvettes were loaded with 1.45 ml of assay temperature-equilibrated MRB and continuously stirred for homogenous O_2 distribution. Later, 100 µl of mitochondrial suspension containing 2-3.5 mg protein were introduced into the cuvettes followed by CI substrates (5 mM malate and 5 mM glutamate) and continuously stirred. The maximal rate of CI respiration (state 3) was evoked by addition of 200 nmoles ADP. The state 3 eventually transitioned to basal (state 4) respiration upon depletion of the ADP. Then 0.5 µM rotenone (CI inhibitor) and 5 mM succinate (CII substrate) were introduced followed by addition of 200 nmoles of ADP to measure CII driven respiration. When CII state 3 eventually transitioned to state 4, 25 µM malonate (CII inhibitor), 3 µM reduced decylubiquinone (CIII substrate: decylubiquinol, reduced by addition of potassium borohydride) and 200 nmoles of ADP were introduced to measure CIII-driven respiration. After achieving CIII state 4, 20 nM antimycin A (CIII inhibitor), CIV substrates (5 mM ascorbate and 200 µM tetramethyl-p-phenylenediamine, TMPD) and 200 nmoles of ADP were added allowing the measurement of CIV-supported respiration. Lastly, oligomycin (2.5 µg/ml), an inhibitor of CV, was added to measure CIV state 4_{ol}. All rates of O_2 consumption were recorded and analyzed using LabPro® software (Qubit Systems) and normalized to mitochondrial protein. The RCR and RCR_{ol} were calculated by dividing respective states 3 and 4 (or 4_{ol}) rates of respiration (Estabrook, 1967) whereas the P/O ratios were calculated as the amount of ADP added divided by the amount of O_2 consumed (as atoms) from the start of state 3 to the point of transition to state 4 (Chance and Williams, 1955).

The effects of Cu at each acclimation temperature for each of the four ETS complexes were investigated using four nominal concentrations (0, 5, 10 and 20 µM) of Cu [as $CuSO_4$. $5H_2O$,

Sigma-Aldrich Oakville, ON]. Each Cu dose was added once at the beginning of CI state 3 and it did not alter the MRB pH. The Cu levels used in my study are well within the range of Cu concentrations measured in liver cytosolic fractions and whole livers of mice and fish (Bunton et al. 1987, Ralle et al. 2010; Kamunde and MacPhail 2011). Importantly, speciation analysis using Visual MINTEQ 3.1 (http://www2.lwr.kth.se/English/ Oursoftware/vminteq/download.html) modified to include Cu complexation by BSA and respiratory substrates revealed that only 0.065% of the total Cu dose existed as Cu^{+} ions. Thus, my exposure dose range as free Cu was 3.25-13 nM which is biologically relevant for studying effects and mechanisms of Cu toxicity in isolated mitochondria.

4.3.5. Mitochondrial respiration and Cu exposure after hypoxia-reoxygenation (HRO)

The measurement of mitochondrial respiration after HRO was done according to the method of Onukwufor et al. (2014). In this method, normoxic CI driven state 3 and 4 respiration rates were first measured as explained above. Because in their intracellular environment mitochondria are exposed to normal O_2 levels in the range of 2.25-3.75 torr (Gnaiger and Kuznetsov, 2002), the MRB was made hypoxic by bubbling N_2 into the cuvettes to attain PO_2 >0<2 torr. The mitochondria were then incubated under hypoxic conditions for 10 min followed by reoxygenation to 100% air saturation. The effect of HRO on CI-IV driven respirations was then measured by sequential ETS inhibition and activation as described above. The duration (10 min) of hypoxia used in this study is typical for mechanistic studies using isolated mitochondria model and took into consideration the potential additive effects of the other two stressors (temperature and Cu). Moreover, some of us (Onukwufor et al. 2014) recently showed that effects of duration of hypoxia on mitochondrial respiration plateau after 30 min. Note that while effects of both

hypoxia and HRO may be assessed in whole fish studies, my isolated mitochondria model permits assessment of effects of only HRO.

Lastly, to assess the combined effects of HRO and Cu, the predetermined doses of the metal (5, 10, and 20 μM) were added after taking the mitochondria through the HRO procedure and CI-IV supported respiration rates were measured in one run as described above. Note that each Cu dose was introduced once during CI active phosphorylation, i.e., after addition of CI substrates (glutamate and malate) and ADP.

4.4. Statistical analysis

The data are expressed as means ± SEM, and the level of significance for all statistical tests was set at $p < 0.05$. All statistical tests were done using Statistica version 6.0 (StatSoft Inc., Tulsa, OK) unless otherwise stated. The data were also tested for normality of distributions (Chi-Square test) and homogeneity of variances (Levene's test) and log transformed when necessary. The effects of acute temperature rise and ETS complex on mitochondrial respiration were analyzed using 2-way ANOVA while those of acclimation temperature, HRO and Cu were analyzed by 3-way ANOVA. Post hoc pairwise comparisons were done using Tukey's HSD test to identify the significantly different means. The body and liver weights, hepatosomatic index (HSI) and mitochondrial protein data were analyzed by 2-tailed Student's *t*-test using Sigma Plot version 10.0 (Systat Software Inc., IL). Graphical figures were drawn using Sigma Plot version 10.0.

4.5. Results

4.5.1. Body and liver weights and mitochondrial protein

Following 8 weeks of warm acclimation, fish body (Fig. 4.2a) and liver (Fig. 4.2b) weights were significantly lower in the warm acclimated compared with the control (cold acclimated) fish. However, the HSI in warm acclimated fish was not statistically different from that of the controls (Fig. 4.2c). Interestingly, liver mitochondrial protein concentration (mg/g liver) was significantly elevated in warm acclimated fish (Fig. 4.2d) and as a result, warm acclimated trout had higher mitochondrial protein/body weight relative to cold acclimated trout.

4.5.2. Effects of acute temperature rise on ETS respiratory function

Measurement of ETS CI to IV respiratory function after acute temperature rise (11→20 ^{o}C) (Fig. 4.3) showed that mitochondria isolated from fish maintained at 11 ^{o}C had highly elevated state 3 (Fig. 4.3a) and 4 (Fig. 4.3b) respiration rates via all four ETS complexes when rates at 20 ^{o}C were compared with those at 11 ^{o}C. These effects were reflected in the highly significant temperature main effect for both state 3 ($F_{1,40}$ = 183, $p < 0.0001$) and 4 ($F_{1,40}$ = 201, $p < 0.0001$). As well, there was a significant main effect of ETS complex wherein both state 3 ($F_{1,40}$ = 37.7, $p < 0.0001$) and 4 ($F_{1,40}$ = 121, $p < 0.0001$) increased from CI to CIV. The increase in state 4 was greater than that of state 3, resulting in reduced RCR (Fig. 4.3c) with both temperature ($F_{1,40}$ = 15, $p = 0.0004$) and ETS complex ($F_{1,40}$ = 44, $p < 0.0001$).

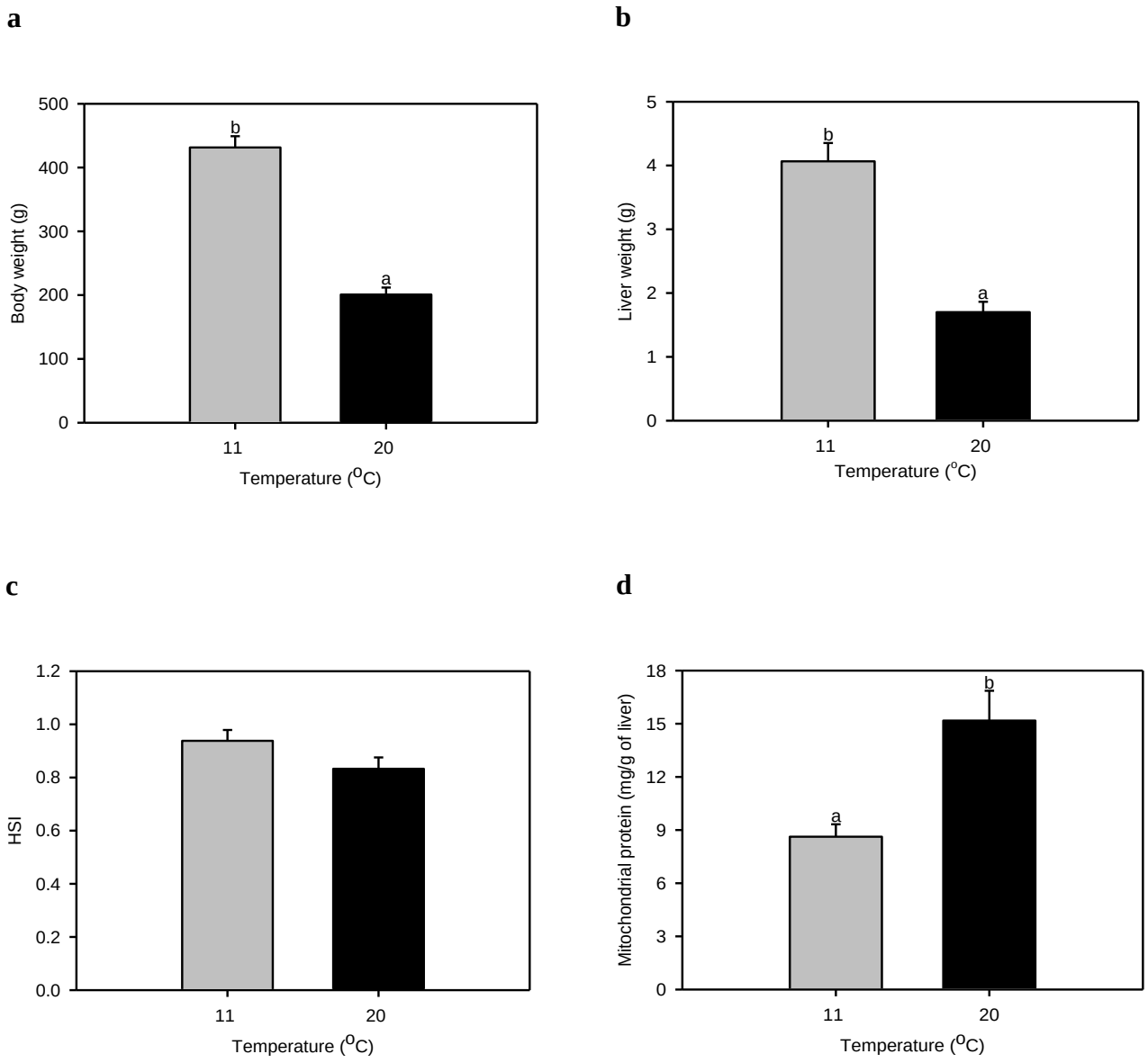

Figure 4.2. Effect of warm acclimation on rainbow trout body (a) and liver (b) weights, hepatosomatic index (c) and mitochondrial protein (d). Data are means ± SEM, n = 5. Different letters indicate significant difference between acclimated and controls, two-tailed Student's *t*-test, $p < 0.05$.

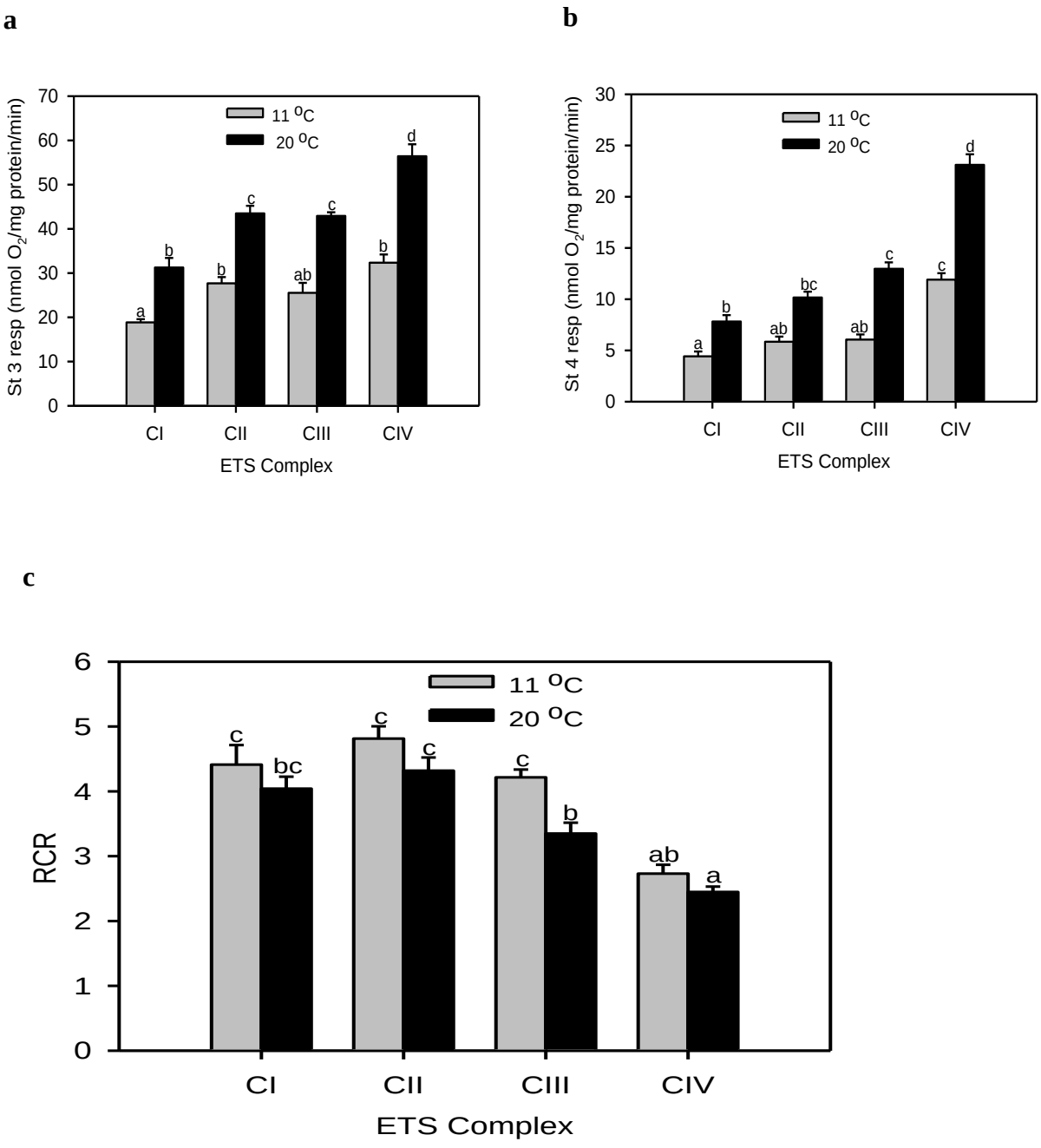

Figure 4.3. Effect of acute temperature rise on rainbow trout liver mitochondria ETS complexes I-IV (CI, CII, CIII, CIV) respiratory activities. a: state 3; b: state 4; c: RCR. Data are means ± SEM, n = 5. Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$.

4.5.3. Effects of warm acclimation, HRO and Cu on CI respiratory function

The summary of main effects and interactions (Table 4.1) shows that warm acclimation, HRO and Cu exposure differently and interactively modulated ETS complex CI-IV respiratory activity. First, CI-driven state 3 measured at 20 ^{o}C was, surprisingly, similar to that of control fish measured at 11 ^{o}C (Fig. 4.4a) despite the 9 ^{o}C difference in assay temperature. For the entire data set, warm acclimation had a significant ($F_{1,64} = 34.3$, $p < 0.0001$) inhibitory effect on CI state 3. Following HRO, CI-driven state 3 was reduced by 51 and 76% in control and warm acclimated mitochondria, respectively, with an overall highly significant ($F_{1,64} = 168$, $p < 0.0001$) inhibitory main effect. Cu exposure on the other hand did not significantly ($F_{3,64} = 1.66$, $p = 0.19$) alter CI state 3 but mitochondria exposed to Cu + HRO had lower state 3 rates than those exposed to Cu alone as affirmed by a significant ($F_{3,64} = 7.03$, $p = 0.0004$) Cu × HRO interaction. All of the other interaction terms on CI state 3 were not significant (Table 4.1). Contrasting the inhibitory effect on CI state 3, warm acclimation increased CI-driven state 4 respiration by 57% in controls (Fig. 4.4b) with an overall significant stimulatory ($F_{1,64} = 5.76$, $p = 0.019$) main effect. Cu exposure also had an overall stimulatory effect ($F_{3,64} = 2.87$, $p = 0.04$) on CI state 4 whereas HRO inhibited ($F_{1,64} = 36.0$, $p < 0.0001$) it. For this metric, the only significant interaction observed was for acclimation × Cu ($F_{1,64} = 19.1$, $p < 0.0001$) in which the stimulatory effect of warm acclimation was increased by Cu. Consistent with their effects on state 3 and 4, CI RCR (Fig. 4.4c) was reduced by warm acclimation ($F_{1,64} = 53.9$, $p < 0.0001$), HRO ($F_{1,64} = 34.0$, $p < 0.0001$) and Cu exposure ($F_{3,64} = 4.89$, $= < 0.004$). Moreover, there were significant Cu × HRO ($F_{3,64} = 7.01$, $p = 0.0004$) and acclimation × HRO ($F_{1,64} = 8.52$, $p = 0.005$) interactions wherein Cu imposed a greater reduction in RCR after HRO, and warm acclimated mitochondria RCR was more severely reduced by HRO. Lastly, CI P/O ratio (Fig. 4d) was

Table 4.1: Main effects and interactions of warm acclimation (Ac), hypoxia-reoxygenation (HRO) and Cu exposure on CI to IV functional traits. Numbers in brackets are p values. Significant F values are bolded and indicate profound effects of warm acclimation and HRO on ETS function.

Complex	Metric	Main Effects and Interactions						
		HRO	***Cu***	***Ac***	***Cu×HRO***	***Ac×HRO***	***Ac×Cu***	***Ac×HRO×Cu***
		$F_{1,64}$ ***(p)***	$F_{3,64}$ ***(p)***	$F_{1,64}$ ***(p)***	$F_{3,64}$ ***(p)***	$F_{1,64}$ ***(p)***	$F_{3,64}$ ***(p)***	$F_{3,64}$ ***(p)***
CI	St 3	**168(<0.0001)**	1.66(0.19)	**34.3(<0.0001)**	**7.03(0.0004)**	0.00(0.95)	0.49(0.69)	2.39(0.08)
	St 4	**36.0(<0.0001)**	**2.87(0.04)**	**5.76(0.019)**	0.53(0.66)	0.26(0.86)	**19.1(<0.0001)**	2.06(0.11)
	RCR	**34.0(<0.0001)**	**4.89(0.004)**	**53.9(<0.0001)**	**7.01(0.0004)**	**8.52(0.005)**	1.27(0.29)	1.96(0.13)
	P/O	**74.8(<0.0001)**	0.67(0.57)	**35.3(<0.0001)**	0.03(0.99)	2.69(0.11)	0.94(0.42)	1.61(0.20)
CII	St 3	**73.0(<0.0001)**	**3.25(0.027)**	**15.0(0.0003)**	**3.22(0.028)**	1.33(0.25)	0.73(0.54)	0.67(0.57)
	St 4	**16.4(0.0001)**	1.40(0.25)	**13.6(0.0005)**	0.37(0.77)	**6.26(0.01)**	**2.77(0.049)**	1.73(0.17)
	RCR	1.63(0.21)	**5.68(0.002)**	**50.5(<0.0001)**	**5.60(0.002)**	0.41(0.53)	**3.16(0.03)**	**3.08(0.03)**
	P/O	**52.2(<0.0001)**	0.23(0.87)	**35.7(<0.0001)**	0.38(0.77)	0.34(0.56)	1.37(0.26)	0.44(0.73)
CIII	St 3	**112(<0.0001)**	**11.3(<0.0001)**	**8.30(0.005)**	2.21(0.095)	**26.6(<0.0001)**	**3.58 (0.019)**	**6.18 (0.0009)**
	St 4	**41.2(<0.0001)**	**6.10(0.001)**	**25.9(<0.0001)**	0.68(0.57)	0.27(0.61)	2.15(0.10)	**6.92(0.0004)**
	RCR	0.66(0.55)	1.79(0.16)	**28.7(<0.0001)**	0.74(0.53)	3.17(0.08)	1.03 (0.38)	1.03(0.39)
	P/O	**28.9(<0.0001)**	0.17(0.91)	**7.65(0.007)**	0.29(0.83)	1.11(0.29)	0.06(0.98)	1.06(0.37)
CIV	St 3	**47.7(<0.0001)**	**6.09(0.001)**	**6.86(0.01)**	0.94(0.43)	**6.20(0.015)**	**4.73(0.005)**	0.91(0.44)
	St 4	**14.6(0.0003)**	1.67(0.18)	**28.9(<0.0001)**	1.16(0.33)	**6.88(0.01)**	1.74(0.17)	1.15(0.34)
	RCR	**11.1(0.001)**	2.38(0.08**)**	**5.97(0.017)**	**3.41(0.02)**	**45.9(<0.0001)**	**5.17(0.003)**	1.29(0.29)
	P/O	**67.7(<0.0001)**	**4.09(0.01)**	**64.4(<0.0001)**	2.73(0.051)	0.53(0.47)	0.68(0.57)	1.23(0.31)

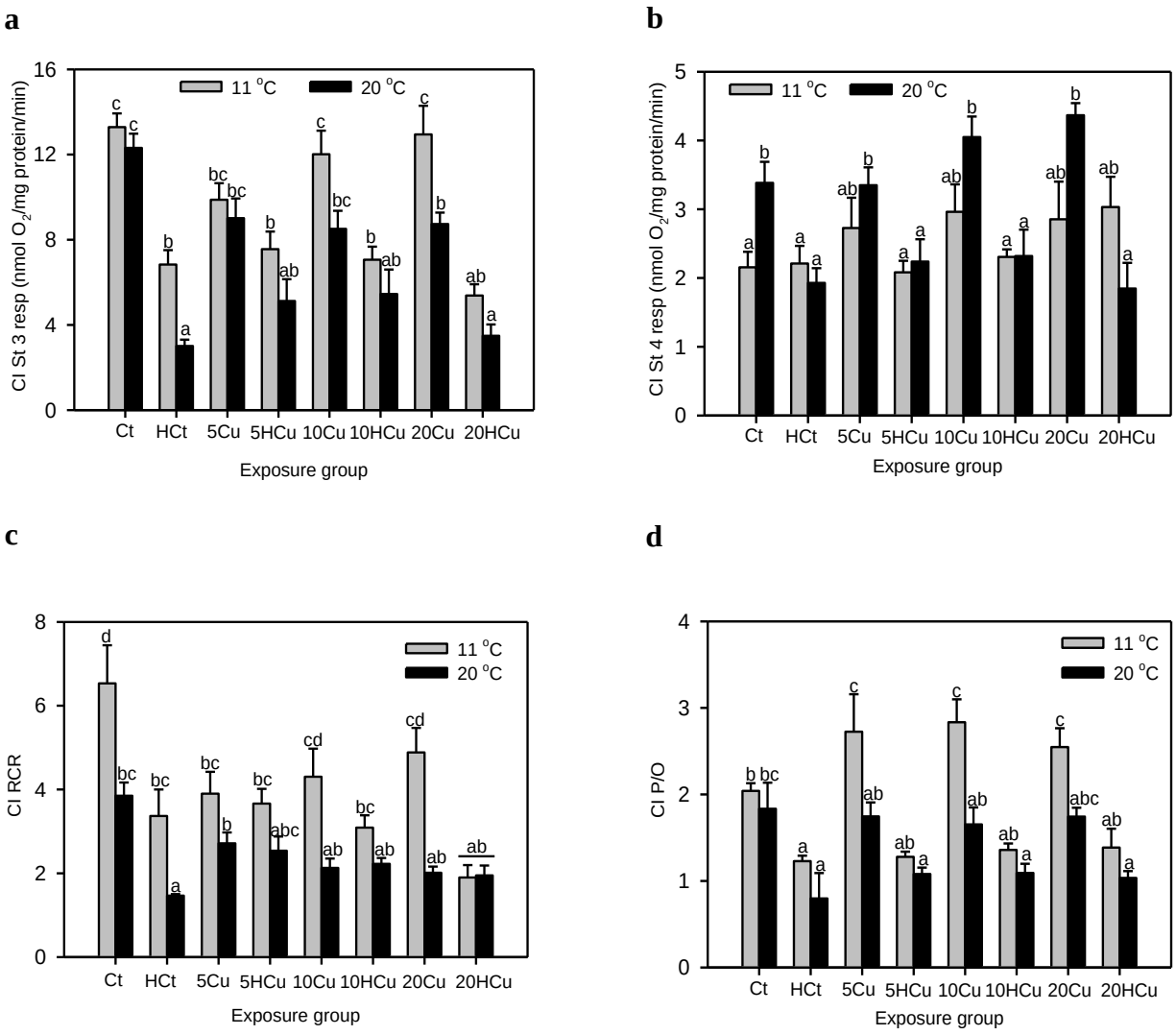

Figure 4.4. Effect of warm acclimation, Cu exposure and HRO on rainbow trout liver mitochondria ETS complex I respiratory activity. a: state 3; b: state 4; c: RCR; d: P/O ratio. The experimental groups are Ct: controls; HCt: hypoxia-reoxygenation (HRO) control; 5Cu: 5 µm Cu alone; 5HCu: 5 µm Cu + HRO; 10Cu: 10 µm Cu alone; 10HCu: 10 µm Cu + HRO; 20Cu: 20 µm Cu alone; 20HCu: 20 µm Cu + HRO. Data are means ± SEM, n = 5. Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$.

significantly reduced by both warm acclimation ($F_{1,64} = 35.32$, $p < 0.0001$) and HRO ($F_{1,64} = 74.76$, $p < 0.0001$). Cu exposure alone increased the P/O ratio in the 11 ^{o}C acclimated but reduced it in the 20 ^{o}C acclimated mitochondria resulting in an overall statistically not significant ($F_{3,64} = 0.67$, $p = 0.57$) main effect. There were no significant interactions among the stressors on CI P/O ratio (Table 4.1).

4.5.4. Effects of warm acclimation, HRO and Cu on CII respiratory function

Warm acclimation ($F_{1,64} = 15.0$, $p = 0.0002$), HRO ($F_{1,64} = 73.0$, $p < 0.0001$) and Cu exposure ($F_{3,64} = 3.25$, $p = 0.027$) all reduced CII-driven state 3 (Fig. 4.5a) with HRO imposing a massive 71% reduction in respiration in warm acclimated mitochondria. There was a significant Cu × HRO interaction ($F_{3,64} = 3.22$, $p = 0.028$) wherein CII state 3 was lower in HRO + Cu treatments compared with the Cu alone treatments. None of the other interactions terms on CII state 3 were significant (Table 4.1). As shown in Fig. 4.5b, CII-supported state 4 was stimulated ($F_{1,64} = 13.6$, $p = 0.0005$) by warm acclimation and reduced by HRO ($F_{1,64} = 16.4$, $p = 0.0001$) while Cu exposure had no overall effect ($F_{3,64} = 1.40$, $p = 0.25$). Significant 2-way interactions for acclimation × HRO ($F_{1,64} = 6.26$, $p = 0.015$) and acclimation × Cu ($F_{3,64} = 2.77$, $p < 0.05$) on this parameter were observed. With regards to CII RCR (Fig. 4.5c), a 40% reduction was observed in controls after warm acclimation with an overall highly significant ($F_{1,64} = 50.5$, $p < 0.0001$) main effect of this experimental factor. Cu exposure also significantly ($F_{3,64} = 5.67$, $p = 0.002$) reduced the RCR whereas HRO ($F_{1,64} = 1.63$, $p = 0.21$) did not alter it. The main effect interactions on CII RCR were significant for Cu × HRO ($F_{3,64} = 5.60$, $p = 0.002$), acclimation × Cu ($F_{3,64} = 3.16$, $p = 0.03$) and acclimation × HRO × Cu ($F_{3,64} = 3.08$, $p = 0.03$). Lastly, CII P/O ratios (Fig. 4.5d) in warm acclimated were consistently lower than those of the cold acclimated mitochondria consistent with the significant ($F_{1,64} = 35.7$, $p < 0.0001$) main effect of acclimation. Moreover,

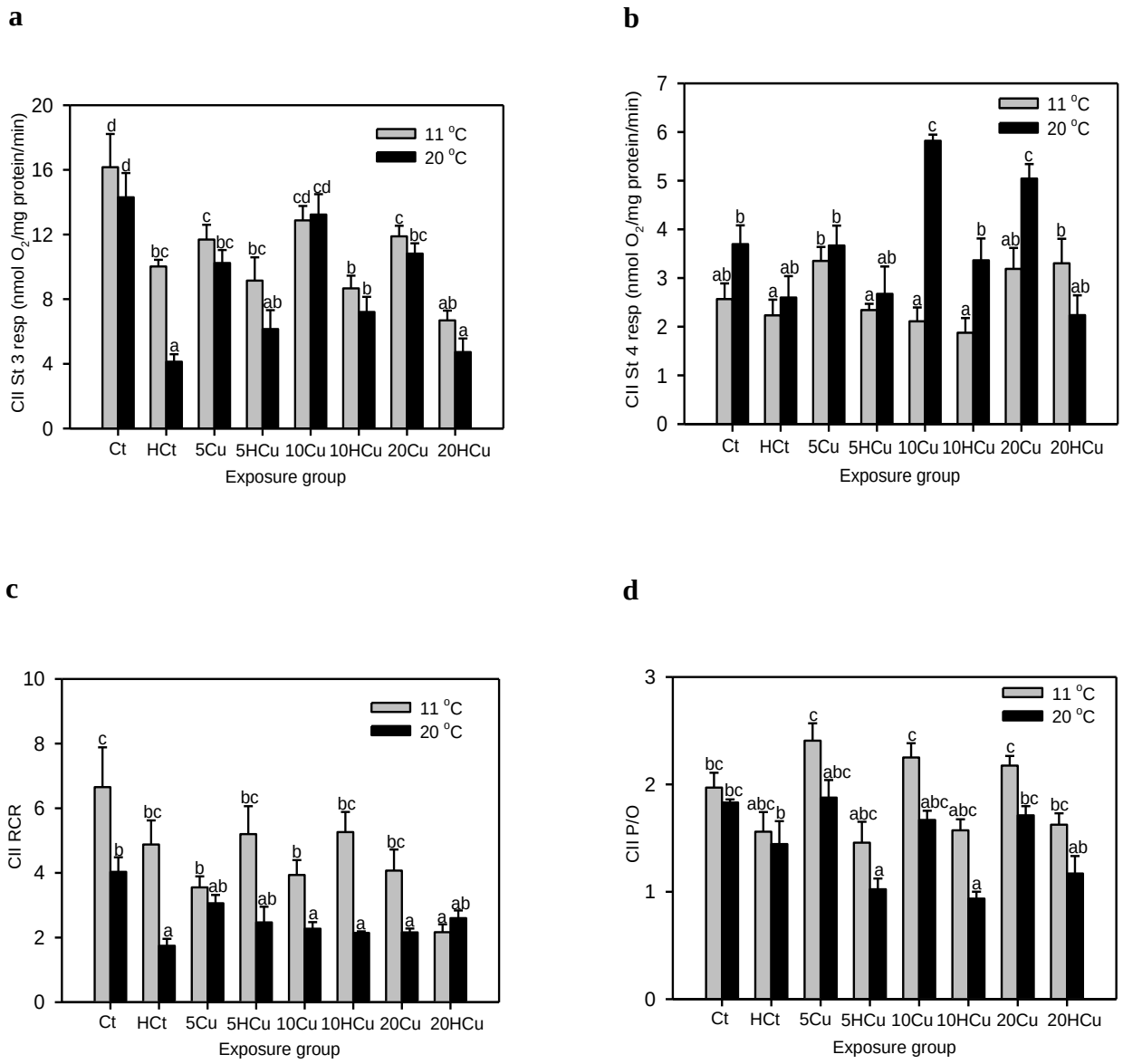

Figure 4.5. Effect of warm acclimation, Cu exposure and HRO on rainbow trout liver mitochondria ETS complex II respiratory activity. a: state 3; b: state 4; c: RCR; d: P/O ratio. The experimental groups are Ct: controls; HCt: hypoxia-reoxygenation (HRO) control; 5Cu: 5 µm Cu alone; 5HCu: 5 µm Cu + HRO; 10Cu: 10 µm Cu alone; 10HCu: 10 µm Cu + HRO; 20Cu: 20 µm Cu alone; 20HCu: 20 µm Cu + HRO. Data are means ± SEM, n = 5. Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$.

HRO highly significantly reduced CII P/O ratios ($F_{1,64} = 52.2$, $p < 0.0001$) whereas the effect of Cu exposure was not significant ($F_{3,64} = 0.23$, $p = 0.87$). The interaction terms on this metric were all not significant (Table 4.1).

4.5.5. Effects of warm acclimation, HRO and Cu on CIII respiratory function

The measured CIII respiratory functional indices (Fig. 4.6a) indicate that state 3 was significantly reduced by warm acclimation ($F_{1,64} = 8.30$, $p = 0.005$), HRO ($F_{1,64} = 112$, $p < 0.0001$) and Cu exposure ($F_{3,64} = 11.3$, $p < 0.0001$). Importantly, significant interactions between acclimation × HRO ($F_{1,64} = 26.6$, $p < 0.0001$), acclimation × Cu ($F_{3,64} = 3.58$, $p = 0.019$) and acclimation × HRO × Cu ($F_{3,64} = 6.18$, $p = 0.0009$) were observed. For CIII state 4, significant overall inhibitory effects of both HRO ($F_{1,64} = 41.2$, $p < 0.0001$) and Cu exposure ($F_{1,64} = 6.10$, $p = 0.001$) were revealed (Fig. 4.6b). The inhibitory effect of HRO was much greater in cold acclimated mitochondria wherein a 64% reduction in state 4 occurred compared with no change in warm acclimated mitochondria. Warm acclimation overall stimulated ($F_{1,64} = 25.9$, $p = 0.0001$) CIII state 4 despite the lower rate in the 20 ^{o}C acclimated controls relative to the 11 ^{o}C controls. Assessment of interactions of the independent variables on CIII state 4 revealed that only the 3-way interaction ($F_{3,64} = 6.92$, $p = 0.0004$) was significant (Table 4.1). Moreover, only acclimation ($F_{1,64} = 28.7$, $p < 0.0001$) evoked an overall significant effect on CIII RCR in which mitochondria from the warm acclimated fish consistently displayed lower RCR values irrespective of the other treatments they received (Fig. 4.6c). The effects of HRO and Cu exposure on CIII RCR, as well as all the interactions, were not significant (Table 4.1). For CIII P/O ratio (Fig. 4.6d), there were overall significant effects of warm acclimation ($F_{1,64} = 7.65$, $p = 0.007$) and HRO ($F_{1,64} = 28.9$, $p < 0.0001$) in which warm acclimated and HRO treated

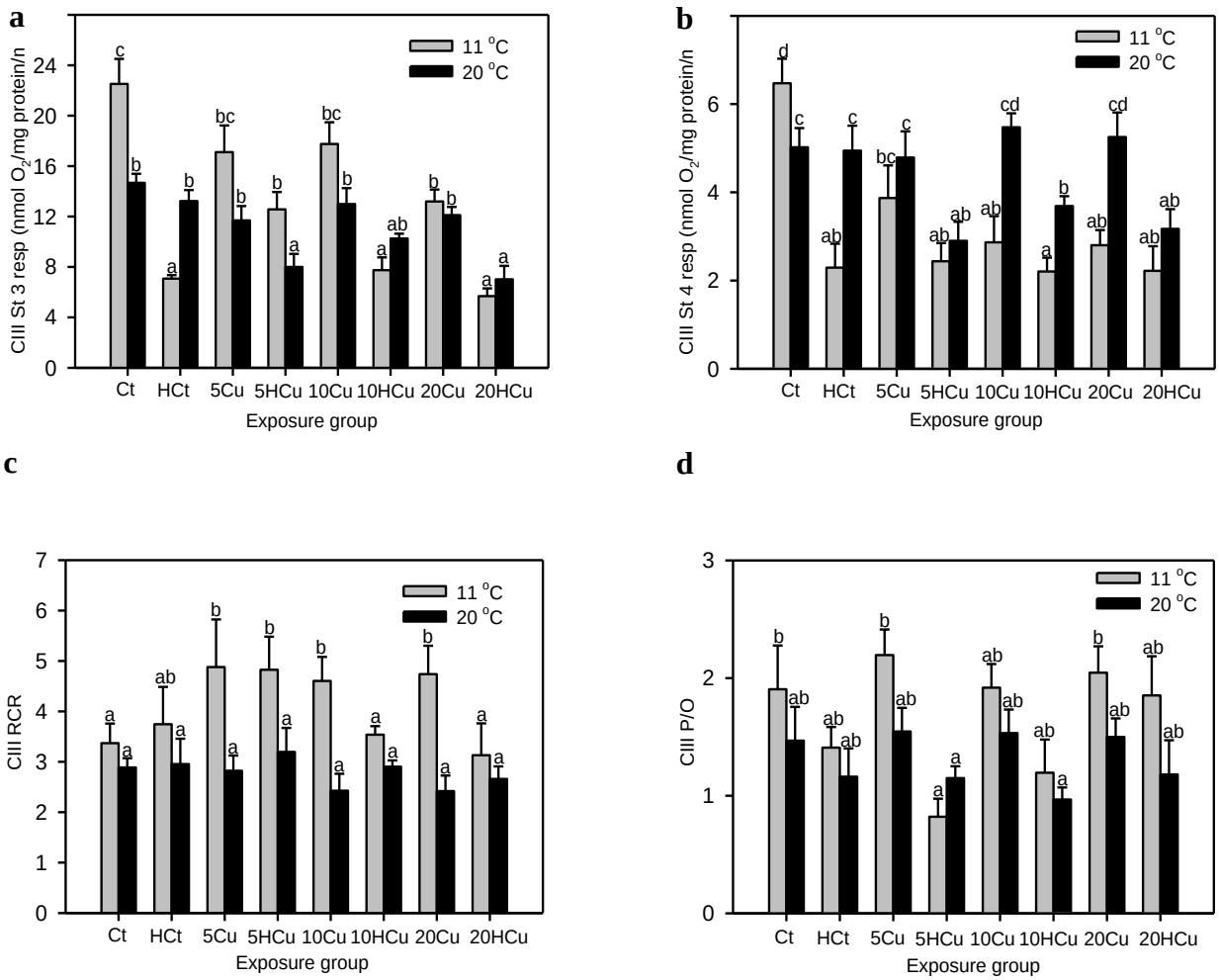

Figure 4.6. Effect of warm acclimation, Cu exposure and HRO on rainbow trout liver mitochondria ETS complex III respiratory activity. a: state 3; b: state 4; c: RCR; d: P/O ratio. The experimental groups are Ct: controls; HCt: hypoxia-reoxygenation (HRO) control; 5Cu: 5 µm Cu alone; 5HCu: 5 µm Cu + HRO; 10Cu: 10 µm Cu alone; 10HCu: 10 µm Cu + HRO; 20Cu: 20 µm Cu alone; 20HCu: 20 µm Cu + HRO. Data are means ± SEM, n = 5. Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$.

mitochondria exhibited lower ratios than those of cold acclimated and HRO un-exposed mitochondria. None of the interaction terms on CIII P/O ratio were significant (Table 4.1).

4.5.6. Effects of warm acclimation, HRO and Cu on CIV respiratory function

Warm acclimation ($F_{1,64} = 6.86$, $p = 0.01$), HRO ($F_{1,64} = 46.7$, $p < 0.0001$) and Cu exposure ($F_{3,64} = 6.09$, $p = 0.001$) reduced CIV (Fig. 4.7a) state 3 with significant interactions terms for acclimation × HRO ($F_{1,64} = 6.20$, $p = 0.015$) and acclimation × Cu ($F_{3,64} = 4.73$, $p < 0.005$). In contrast, CIV state 4 was recalcitrant ($F_{3,64} = 1.67$, $p = 0.18$) to Cu exposure under all conditions but was stimulated ($F_{1,64} = 28.9$, $p < 0.0001$) by warm acclimation and inhibited ($F_{1,64} = 14.6$, $p = 0.0003$) by HRO (Fig. 4.7b). A significant ($F_{1,64} = 6.88$, $p = 0.01$) interaction term for acclimation × HRO was evidence that HRO reduced the stimulatory effect of warm acclimation on CIV state 4.. For CIV RCR (Fig. 4.7c) I found that it was reduced by both warm acclimation ($F_{1,64} = 5.97$, $p = 0.017$) and HRO ($F_{1,64} = 11.1$, $p = 0.001$) but not altered by Cu exposure ($F_{3,64} = 2.38$, $p = 0.08$). Additionally, significant interaction terms were observed for Cu × HRO ($F_{3,64} = 3.41$, $p = 0.02$), acclimation × HRO ($F_{1,64} = 45.9$, $p < 0.0001$) and acclimation × Cu ($F_{3,64} = 5.17$, $p = 0.003$). Lastly, for CIV P/O ratio (Fig. 4.7d) warm acclimation ($F_{1,64} = 64.4$, $p < 0.0001$), HRO ($F_{1,64} = 67.7$, $p < 0.0001$) and Cu exposure ($F_{3,64} = 4.09$, $p = 0.01$) all individually reduced it without significant interactions (Table 4.1).

My sequential inhibition-activation protocol permitted the measurement of state 4_{ol} for CIV only (Fig. 4.8a). The results indicated that warm acclimation ($F_{1,64} = 0.25$, $p = 0.62$) had no effect whereas HRO ($F_{1,64} = 25.9$, $p < 0.0001$) and Cu ($F_{3,64} = 3.17$, $p = 0.03$) both reduced CIV state 4_{ol}, with the only significant interaction being for acclimation × Cu ($F_{3,64} = 3.21$, $p < 0.03$). The related CIV RCR_{ol} (Fig. 4.8b) was altered only by warm acclimation ($F_{1,64} = 12.4$, $p < 0.001$)

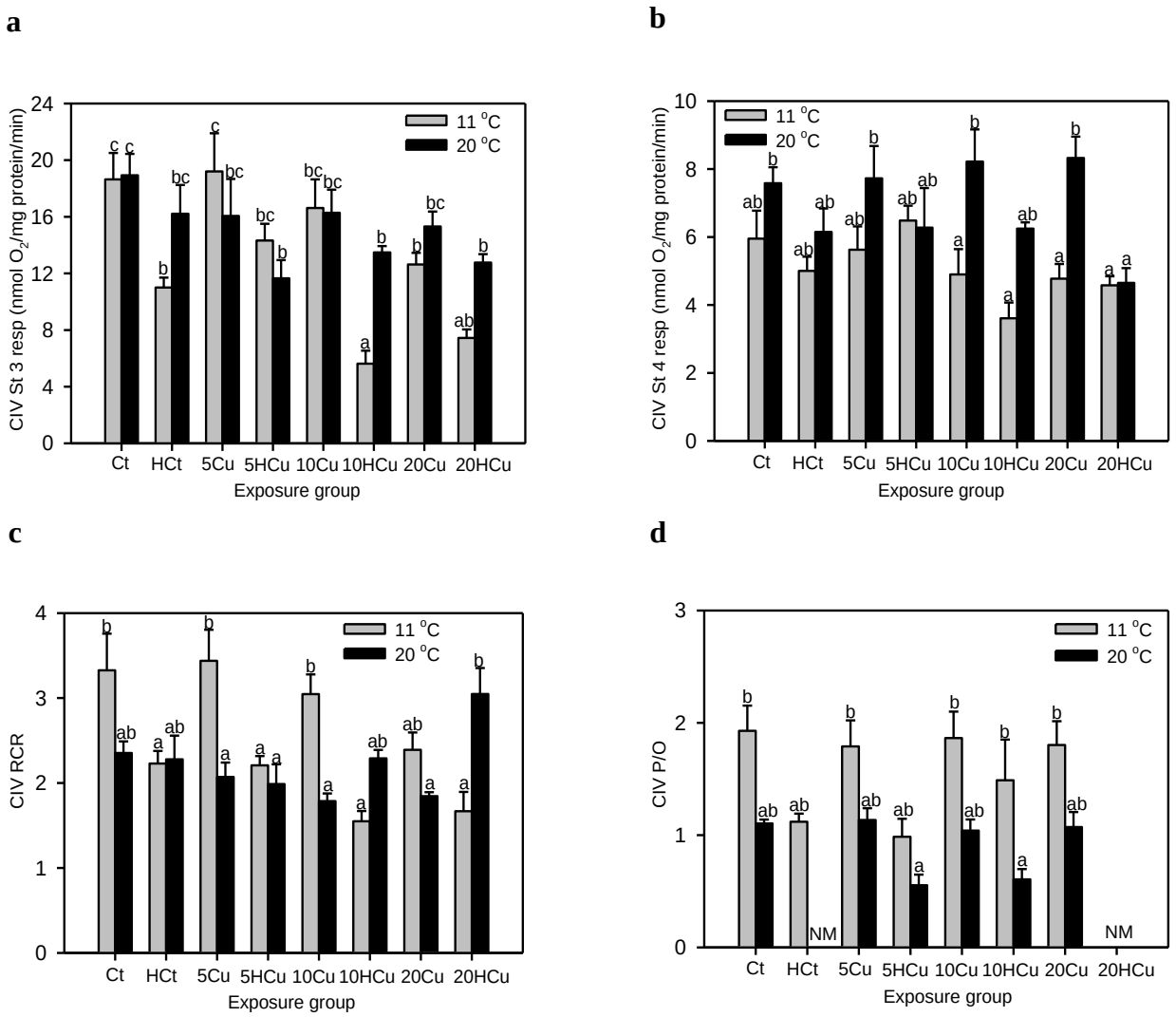

Figure 4.7. Effect of warm acclimation, Cu exposure and HRO on rainbow trout liver mitochondria ETS complex IV respiratory activity. a: state 3; b: state 4; c: RCR; d: P/O ratio. The experimental groups are Ct: controls; HCt: hypoxia-reoxygenation (HRO) control; 5Cu: 5 µm Cu alone; 5HCu: 5 µm Cu + HRO; 10Cu: 10 µm Cu alone; 10HCu: 10 µm Cu + HRO; 20Cu: 20 µm Cu alone; 20HCu: 20 µm Cu + HRO. Data are means ± SEM, n = 5. Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$.

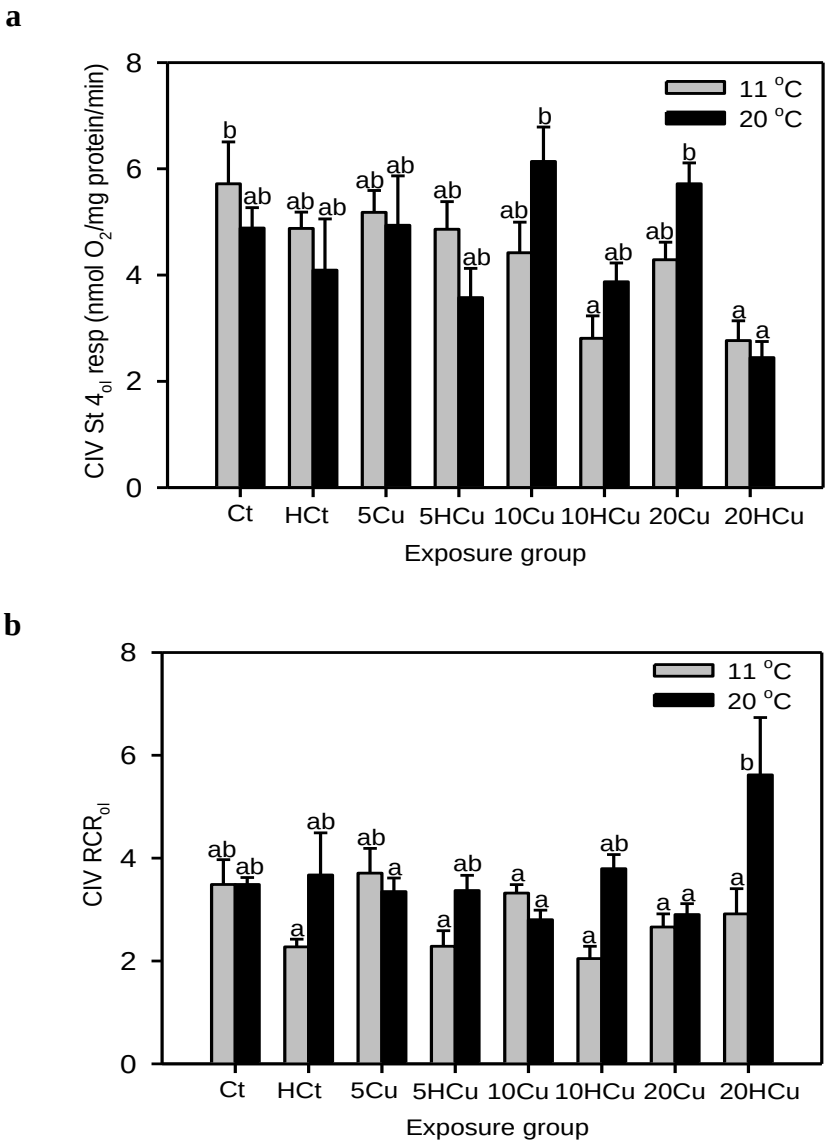

Figure 4.8. Effect of warm acclimation, Cu exposure and HRO on rainbow trout liver mitochondria ETS complex IV state 4_{ol} (a) and RCR_{ol} (b). The experimental groups are Ct: controls; HCt: hypoxia-reoxygenation (HRO) control; 5Cu: 5 µm Cu alone; 5HCu: 5 µm Cu + HRO; 10Cu: 10 µm Cu alone; 10HCu: 10 µm Cu + HRO; 20Cu: 20 µm Cu alone; 20HCu: 20 µm Cu + HRO. Data are means ± SEM, n = 5. Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$.

without significant effects of either HRO ($F_{1,64} = 0.02$, $p = 0.89$) or Cu exposure ($F_{3,64} = 0.97$, $p = 0.41$). For this metric, significant interactions were observed for Cu × HRO ($F_{1,64} = 4.94$, $p = 0.004$) and acclimation × HRO ($F_{1,64} = 17.8$, $p < 0.0001$).

4.6. Discussion

While the basis of temperature and hypoxia as interlinked stressors in aquatic organisms has been justified (Pörtner 2010; Pörtner and Lannig 2009; McBryan et al. 2013) much remains unknown about their mechanisms of interactions on mitochondrial function and the moderating effects of concurrent or sequential metals exposure. In my study, the effects of HRO and Cu and their interactions in liver mitochondria isolated from warm acclimated (20 ^{o}C) and cold acclimated (control: 11 ^{o}C) rainbow trout were assessed using a sequential ETS inhibition and activation protocol comparable to multiple substrate-inhibitor titration (Kuznetsov et al. 2008) that permitted measurements of respiratory activities of CI to IV in one run. My results demonstrated that HRO and warm acclimation markedly altered CI-IV respiratory activities while the effects of Cu at the concentrations tested were qualitatively and quantitatively more muted. I revealed that ETS complexes have differential sensitivity to warm temperature acclimation, HRO and Cu, and that 2-and/or 3-way interactions occurred on all functional indices except the P/O ratio. It is noteworthy that while I have discussed all of the main effects, interactions complicate interpretation of the significance of main effects. Therefore where significant interactions were present, the effect of the interaction was deemed the more meaningful result and post hoc pairwise comparisons subsequently delineated the differences among the experimental group means. Overall, the interactions observed suggest that mitochondrial respiratory activity was more vulnerable to Cu in the presence of HRO and/or warm temperature acclimation. Moreover, contrary to my working hypothesis, I found no

evidence of cross-tolerance, i.e., warm acclimation did not reduce the sensitivity of ETS respiratory activity to HRO and/or Cu exposure.

4.6.1. Individual effects of warm temperature, HRO and Cu on mitochondrial respiration

Acute temperature rise stimulated state 3 respiration via all four ETS complexes as previously reported for CI (Sappal et al. 2014a; Iftikar and Hickey, 2013; Onukwufor 2015), CII (Sappal et al. 2014b; Iftikar and Hickey 2013), CIII (Strobel et al. 2013) and CIV (Iftikar and Hickey 2013) in mitochondria from a variety of fish tissues. High mitochondrial respiration rates following acute temperature rise (within a species thermal tolerance range) have been attributed to alterations of the substrate oxidation subsystem (Dufour et al. 1996; Chamberlin 2004) and increased diffusion rates (Guderley and St-Pierre 2002). In contrast, warm acclimation decelerated the maximal respiration rates supported by all the four ETS complexes with the order of inhibition being CI > CII > CIII > CIV. Notably, I observed reduction in respiration though the assays were done at the respective acclimation temperatures with a 9 ^{o}C differential. Consistent with my findings, Guderley and Johnston (1996) and Bouchard and Guderley (2003) reported that CI maximal respiration rates of mitochondria isolated from warm acclimated short-nosed sculpin (*Myoxocephalus scorpius*) and rainbow trout muscle at warm temperature were comparable to the rates at cold temperature for cold acclimated muscle mitochondria.

Concurrent with the inhibition of maximal respiration, the basal respiration rates via all four complexes were stimulated by warm acclimation following an inverse rank order (CIV > CIII > CII > CI) to that of maximal respiration. As a result, warm acclimated mitochondria exhibited reduced oxidation capacities and coupling efficiencies. Importantly, the mitochondrial inefficiency in warm acclimated fish apparently compromised energy metabolism leading to

reduced body and liver weights (Fig. 4.2), although other potential causes such as dietary limitation and increased energy demand to handle chronic thermal stress cannot be ruled out. Indeed, the observed increase in mitochondrial protein content per gram liver may be indicative of increased synthesis of defense proteins, e.g., HSP, MTs and proteins of the antioxidant system, inasmuch as reduced water content at warm temperature as shown for carp (*Cyprinus carpio*) muscle (Heap et al. 1986) also can result in increased mitochondrial protein content per gram liver weight. Contrastingly, during cold compensation, increased liver mitochondrial protein content occurs in tandem with increased organ mass and does not alter mitochondrial protein per gram liver (Lannig et al. 2005). Note that mitochondrial content based on total protein used here is not as accurate as estimates based on activities of mitochondrial marker enzymes such as citrate synthase and COX (Larsen et al. 2012).

Two elegant time-course studies of warm acclimation in rainbow trout (Bouchard and Guderley 2003, Kraffe et al. 2007) reported biphasic responses of muscle mitochondria CI-supported respiration in which both state 3 and 4 increased in the early phase of acclimation and returned to pre-acclimation levels after a longer period (8 weeks) of acclimation. In my study, state 3 was clearly inhibited whereas state 4 was clearly stimulated after 8 weeks of warm acclimation. This discrepancy may be explained by differences in the acclimation temperature as well as the tissue of mitochondria origin. In this regard, liver and muscle mitochondria from juvenile alligator (*Alligator mississippiensis*) were reported to respond differently to warm acclimation (Guderley and Seebacher 2011). Because I observed global inhibition of the ETS, I posited that warm acclimation altered a fundamental component of the mitochondria such as the membrane structure and function as previously reported (Kraffe et al. 2007 and references therein) either by HVA (Hazel 1995; Guderley, 2004; Moyes and Ballantyne 2011) and/or oxidative stress (Heise

et al. 2006, 2007). Other potential warm acclimation imposed effects that would decelerate the ETS include increased ADP affinity and decreased levels of ANT and ETS complexes (Guderley and St-Pierre 2002; Kraffe et al. 2007; Guderley 2011).

Among the stressors I tested, HRO had the most profound effect on mitochondrial oxidation capacity, with reduction in maximal respiration exceeding 70% and the order of sensitivity being CI > CIII > CII > CIV. Reduced maximal mitochondrial respiration following ischemia/hypoxia and reperfusion is commonly observed in CI-powered mitochondria (Blomgren et al. 2003; Boengler et al. 2007; Shiva et al. 2007; Solaini and Harris 2005; Di Lisa et al. 2011; Maruyama et al. 2013). Moreover, reduction in mitochondrial respiratory function after HRO has been observed after whole animal exposure of the hypoxia-sensitive shovelnose ray, *Aptychotrema rostrata* (Hickey et al. 2012) and the hypoxia-resistant eastern (*Crassostrea virginica)* and pacific oysters (*Crassostrea gigas)* (Ivanina et al. 2012; Sussarellu et al. 2013) suggesting that HRO evokes comparable responses both *in vivo* and *in vitro.* However, lack of effect of HRO on state 3 also has been reported for CII and IV in mammalian mitochondria from a variety of tissues (Weinberg et al. 2000; Bosetti et al. 2004; Solaini and Harris 2005) while stimulation has been observed for C1, II and IV in the eastern oyster gill mitochondria (Kurochkin et al. 2009), and for CII in rat heart mitochondria (Lim et al. 2002; Solaini and Harris 2005; Maruyama et al. 2013). Consistent with these apparently ETS complex-specific effects on mitochondrial respiration, enzyme activities of CII and IV (Rouslin 1983; Solaini and Harris 2005) remained stable while those of CI and III (Rouslin 1983; Schild et al. 1997; Onukwufor et al. 2014) were inhibited by HRO. Interestingly, in my study HRO also reduced CI:CII respiration ratio (data not shown) in mitochondria from the warm acclimated trout. This is an indication of reduced OXPHOS efficiency because unlike CI, CII does not pump protons outwardly across the inner

mitochondrial membrane and therefore its increased contribution to respiration results in lower H^+/e^- stoichiometry (Wojtovich et al. 2013).

Contrasting the stimulatory effect of warm acclimation, HRO reduced basal respiration supported by the four ETS complexes with the severity of inhibition being CIII > CI > CII > CIV. Previous studies on the effect of HRO on state 4 have generated variable results ranging from inhibition (Weinberg et al. 2000; Sussarellu et al. 2013) and stimulation (Schild et al. 2003; Bosetti et al. 2004; Onukwufor et al. 2014) to absence of effect (Maruyama et al. 2013). These disparities may in part result from differences in experimental conditions, particularly temperature and duration of hypoxia and/or reoxygenation, or species of mitochondrial origin and their sensitivities to hypoxia. In this regard, state 4 of CI, II and IV in isolated gill mitochondria of the hypoxia-resistant eastern oysters measured at different times of reoxygenation after hypoxia (anoxia), were all elevated in the early phase (1-6 h) but returned to control levels after a longer (12 h) duration of reoxygenation (Kurochkin et al. 2009). In contrast, the inhibition of rat liver mitochondria CI state 3 worsened with time of both hypoxia and reoxygenation (Schild et al. 1997) while duration of hypoxia exacerbated inhibition of state 3 and stimulation of state 4 in rainbow trout liver mitochondria (Onukwufor et al. 2014).

The global nature of the HRO-imposed reduction in ETS respiratory activity observed in my study suggests that HRO affected mitochondrial components fundamental to their function and/or acted via non-specific mechanisms. I speculate that oxidative stress is a possible mechanism because ROS can act indiscriminately to cause large-scale impairment of respiratory function and mitochondria exhibit increased rates of ROS production following HRO (Caraceni et al. 1995; Korge et al. 2008). My finding that mitochondrial oxidation supported by CI and III, the main sites of ROS production in the mitochondria (Murphy, 2009), were the most impaired

by HRO lends support to an oxidative stress mode of action. On the other hand, the resistance of CIV to HRO, and indeed to other stressors, could be explained by the existence of an excess capacity of this enzyme (Antunes et al. 2004; Blier et al. 2014), a feature that is important in maintaining the ETS in oxidized state.

Cu exposure evoked erratic and muted effects on mitochondrial ETS respiratory activity, possibly because relatively low doses of the metal were used. The dose range I tested was narrow because the sequential ETS inhibition-activation protocol resulted in progressively longer Cu exposure durations for the distal ETS complexes. Moreover, I showed that the RCR decreased from CI to CIV and that >20 μM Cu with HRO during a sequential ETS inhibition-activation assay completely uncoupled CIV OXPHOS. Overall, while Cu did not affect CI maximal respiration, it inhibited those of CII, III and IV with a rank order of CIII = CIV > CII. Previous studies reported that Cu (at higher concentrations) inhibits CI and/or II maximal respiration in mitochondria isolated from livers of rat (Saris and Skulskii 1991; Belyaeva et al. 2004, 2011) and fish (Sappal et al. 2014a,b) and gills of bivalves (Collins et al. 2010; Ivanina and Sokolova 2013). Here I show that rainbow trout liver mitochondrial maximal respiration rates for CIII and IV are also inhibited by Cu and that high metal dose increased CI:CII ratio in cold acclimated animals (data not shown) indicative of increased OXPHOS efficiency. Additionally and in part consistent with my recent findings (Sappal et al. 2014a,b), Cu stimulated state 4 of CI and III without affecting CII and IV. Collectively, the effects of Cu on mitochondrial respiratory activity were dependent on the metal dose and the ETS complex.

4.6.2. Effects of warm acclimation, HRO and Cu on coupling and phosphorylation efficiencies

My study showed that while coupling efficiencies of CI-IV were reduced by at least one of the three stressors, the mechanisms varied with the complex and stressor. For example, although HRO and warm acclimation both reduced CI and IV RCR, the former acted primarily by inhibiting state 3 while the latter mainly stimulated state 4. However, because warm acclimation also concurrently reduced state 3, its impact on RCR was much greater relative to that of HRO. While RCR is useful for assessing overall mitochondrial function, I observed here (for CII and III) and previously (Sappal et al. 2014a,b) that RCR can remain unchanged or increase despite reduced respiratory capacity. This typically occurs when state 3 and 4 change in parallel or when state 4 is preferentially inhibited relative to state 3, and can be viewed as a mechanism of preserving OXPHOS efficiency. Nonetheless, in mitochondria from several animal species acute temperature rise *in vitro* reduced RCR for CI (Dufour et al. 1996; Abele et al. 2002; Lemieux et al. 2010; Iftikar and Hickey 2013; Onukwufor et al. 2015), CII (Sokolova 2004) and CIV (Iftikar and Hickey 2013) while acclimation to elevated temperature reduced CI and II RCR (Guderley and Johnston 1996; Bouchard and Guderley, 2003; Strobel et al. 2013). For HRO, reduced CI (Solani et al. 2005, Pravdic et al. 2009; Maruyama et al. 2013) and CII (Shiva et al. 2007; Pravdic et al. 2009) as well as increased CII (Maruyama et al. 2013) RCR have been reported, with the discrepancies being attributed in part to differences in experimental conditions.

The P/O ratios for the four ETS complexes were reduced by HRO more severely than by either warm acclimation or Cu exposure. Here, CIV P/O ratio was the most impacted, likely a reflection of the inherently higher state 4 relative to state 3 for this complex. While measurements of effects of HRO on P/O ratios for all four complexes are few, previous studies on CI and II (Du et al. 1998; Onukwufor et al. 2014) are consistent with my findings. For

temperature effects, my results are consistent with several earlier reports that phosphorylation efficiencies decrease following a substantial acute and chronic temperature rise *in vitro* or *in vivo* (Dufour et al. 1996; Portner et al. 1999a; Abele et al., 2002; Sokolova, 2004) but increase (Lemieux et al. 2010; Sappal et al. 2014b; Onukwufor et al. 2015) or remain unchanged (Gurderley and Johnston 1996; Strobel et al. 2013; Rodnick et al. 2014) following direct and immediate exposure or acclimation to mildly elevated temperatures. Additionally, warm temperature-induced changes may be transitory (Bouchard and Guderley 2003; Kraffe et al. 2007) wherein P/O ratios are elevated early in the course of warm acclimation and return to control levels with longer period of acclimation. Lastly, Cu reduced P/O ratio of CIV only which suggests that this complex might be more sensitive to alterations in Cu levels possibly because it is a cuproenzyme (Zeng et al. 2007). Alternatively, this finding may be a reflection of the longer duration of Cu exposure for CIV during my sequential inhibition and activation protocol. Although the P/O ratios for the other complexes were not altered, Sappal et al. (2014b) recently reported that Cu increased CI P/O ratio, a discrepancy that could be explained by differences in the Cu doses and temperature regimes used.

4.6.3. Interactions of warm acclimation, Cu and HRO on ETS respiratory activity

My study specifically sought to uncover the interactive effects of warm acclimation, Cu and HRO and evidence for cooperative action (synergism and additivity), antagonism and cross-tolerance (Crain et al. 2008; Todgham and Stillman 2013) on mitochondrial respiratory function. I found that while the interactive responses depended on the stressor combinations, mitochondrial metric and ETS complex, additive action was the most common interaction. Overall, the interactions were more commonly exhibited by RCR, highlighting the power of RCR in integrating effects of multiple stressors on mitochondrial function. While I am not aware

of other studies of interactions of Cu with HRO and thermal stress on mitochondrial bioenergetics in fish, a recent *in vivo* study (Mustafa et al. 2012) showed enhancement of toxicity on non-mitochondrial endpoints when the common carp was exposed to dietary Cu under hypoxic relative to normoxic conditions. Similarly, heat stress and Cu exposure increased the toxicity of each other and reciprocally impaired adaptive mechanisms mounted against either stressor in fathead minnows, *Pimephales promelas* (Lapointe et al. 2011). Interestingly, although acclimation to low O_2 levels was shown to increase tolerance to both hypoxia (Rees et al. 2001) and temperature (Burleson and Silva 2011) I did not find increased tolerance to HRO or Cu in liver mitochondria isolated from warm acclimated fish. To the contrary, warm acclimated mitochondria were more sensitive to HRO and Cu.

4.7. Conclusions

Warm acclimation blunted the sensitivity of the ETS to acute temperature rise and imposed global changes on mitochondrial bioenergetics characterized by inhibition of maximal respiration and stimulation of basal respiration that severely uncoupled OXPHOS supported by CI-IV. These changes came at an energetic cost manifested as reduced body and liver weights. While HRO profoundly reduced both maximal and basal respiration similarly uncoupling OXPHOS for all the complexes except CII, the concurrent reduction in basal respiration (proton leak) likely abrogated, at least in part, the loss of OXPHOS efficiency. Cu exposure evoked muted and variable effects exemplified by weakly significant inhibition of CII-CIV maximal respirations without effect on CI, and stimulation of CI and III basal respirations without effect on CII and CIV. Importantly, while warm acclimation-Cu-HRO interactions depended on the stress factor combinations, ETS complex and the mitochondrial metric, I demonstrated that only CIII maximal and basal maximal respiration rates and CII RCR portrayed significant 3-way

interactions. The 2-way interactions of warm acclimation either with Cu or HRO were depicted mostly by CIV, whereas CI and II were the most responsive to combined HRO and Cu exposure. Overall, I revealed for the first time the relative sensitivity of mitochondrial ETS segments to temperature stress, Cu and HRO, and showed that these stressors act additively to impair ETS respiratory activity.

CHAPTER 5

EFFECTS OF COPPER, HYPOXIA AND ACUTE TEMPERATURE SHIFTS FOLLOWING WARM ACCLIMATION ON MITOCHONDRIAL OXIDATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Sappal R, Fast M, Stevens D, Kibenge F, Siah A and Kamunde C. 2015b. Effects of copper, hypoxia and acute temperature shifts following warm acclimation on mitochondrial oxidation in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology. MS# AQTOX-D-15-00327.

5.1 Abstract

Temperature fluctuations, hypoxia and metals pollution frequently occur simultaneously or sequentially in aquatic systems and their interactions may confound interpretation of their biological impacts. With a focus on energy homeostasis, the present study examined how warm acclimation influences the responses and interactions of acute temperature shift, hypoxia and Cu exposure in fish. Rainbow trout (*Oncorhynchus mykiss*) were acclimated to cold (11 ^{o}C; control) and warm (20 ^{o}C) temperature for 3 weeks followed by exposure to environmentally realistic levels of Cu and hypoxia for 24 h. Measurements of mitochondrial ETS respiratory activity supported by complexes I to IV (CI-IV), plasma metabolites and condition indices were done. Warm acclimation reduced fish condition, induced aerobic metabolism and altered the responses of fish to acute temperature shifts, hypoxia and Cu. Whereas warm acclimation decelerated the ETS and increased the sensitivity of maximal oxidation rates of the proximal (CI and II) complexes to acute temperature shift, it reduced the thermal sensitivity of state 4 (proton leak). Effects of Cu with and without hypoxia were variable depending on the acclimation status and functional index. Notably, Cu stimulated respiratory activity in the proximal ETS segments, while hypoxia was mostly inhibitory and minimized the stimulatory effect of Cu. The effects of Cu and hypoxia were modified by temperature and showed reciprocal antagonistic interaction on the ETS and plasma metabolites, with modest additive actions limited to CII and IV state 4. Overall, my results indicate that warm acclimation came at a cost of reduced ETS efficiency and increased sensitivity to added stressors.

5.2 Introduction

The internal body temperature and consequently rates of biological processes in most fish change directly with environmental temperature. However, each fish species has a specific evolutionarily set temperature range that it tolerates, with reduced performance or death occurring both above and below this thermal range (Fry 1947). Temperature tolerance can nonetheless be altered through acclimation (Fry 1947; Beitinger and Lutterschmidt 2011), a phenomenon that shifts the thermal optima allowing fish to survive at temperature regimes deviant from their preferred thermal window. The mechanisms that mediate the acclimation process include compensation for temperature induced changes on rates of biological processes, most importantly energy metabolism, to restore homeostasis (Hochachka and Somero 2002). As a corollary, mitochondria, the main generators of cellular energy, are thought to be key drivers of acclimation and responses to acute temperature stress (Bouchard and Guderley 2003; Guderley 2004; Lemieux et al. 2010). In this regard, acclimation to cold and warm temperature differentially alters aerobic metabolism wherein levels of enzymes (e.g., COX and citrate synthase) required for mitochondrial substrate oxidation increase and decline, respectively (Bouchard and Guderley 2003; O'Brien 2011). However, the acquisition of thermal tolerance may be associated with impairment of biological function under different conditions and whether or not acclimation is overall beneficial remains unclear.

Fish in their natural environments encounter numerous stressors in addition to temperature shifts, and the effects of these stressors may alter or be altered by thermal acclimation. Among these stressors, hypoxia and metals pollution occur in many aquatic systems and are currently among the most pressing environmental issues worldwide. Hypoxia in aquatic systems may result from natural eutrophication and thermal stratification but global warming and organic pollution have

worsened the problem. Hypoxia in and of itself has profound effects on fish physiology with impairment of mitochondrial function (energy metabolism) being a key effect (Richards 2009). While hypoxia-induced alterations in cellular energy balance may modify or be modified by concurrent or subsequent stressors (Pörtner 2010; Burleson and Silva 2011), the underlying mechanisms are not fully understood. Importantly, temperature and/or hypoxia could also alter the mechanisms induced to counteract effects of chemical contaminants, e.g., metals.

Copper is one of the metals frequently found at elevated concentrations in aquatic environments due to its wide spread use by humans. This trace metal is essential for normal physiology in all organisms, serving as a cofactor in several metalloproteins required for critical cellular functions including aerobic metabolism (Pena et al. 1999). However, when excessive, Cu is toxic because it promotes the production of ROS through redox-cycling, displaces other metals from metalloproteins and/or oxidizes thiol groups in proteins thus impairing their function (Stohs and Bagchi 1995; Glass and Stark 1997; Pena et al. 1999). Notably, there is substantial empirical evidence that Cu accumulates in the mitochondria and disrupts their structure and function (Krumschnabel et al. 2005; Kamunde and MacPhail 2008; Garceau et al. 2010; Sappal et al. 2014a,b). While significant strides have been made in delineating the mechanisms of Cu-induced toxicity, much remains unknown regarding the interactions with other stressors. It has been argued that because of the apparent convergence of the effects of thermal stress, hypoxia and Cu on the mitochondria, these organelles might provide a valuable platform for probing the mechanism of interactions of these stressors (Sappal et al. 2015a). Generally, stressor combinations evoke complex responses in fish (McBryan et al. 2013, Schulte 2014) but the underlying mechanisms remain largely unknown. One hypothesis is that interactive effects/responses occur because stressors act on similar biological mechanisms (Pörtner et al. 2005) but it remains to be

comprehensively tested for metals, temperature and hypoxia, in part because most studies typically investigate one stressor at a time and rarely consider multiple levels of biological organization.

The aim of the present study was to assess how warm acclimation influences the ability of fish to handle individual and joint effects of subsequent acute temperature change, hypoxia and Cu stress. I argued that interactions among these stress factors would modify biological outcomes predictably. I focused on energy metabolism and measured mitochondrial function and apical endpoints indicative of stress and organismal energy status. By measuring effects at multiple levels of biological organization, I provide insights into the mechanisms underlying temperature-hypoxia-Cu interactions on energy metabolism. Moreover, my study underscores the central role of mitochondria in integrating the effects of multiple stressors in fish.

5.3 Material and methods

5.3.1 Fish and acclimation to warm temperature

All procedures that fish were subjected to were consistent with the Canadian Council on Animal Care guidelines as approved by the University of Prince Edward Island Animal Care Committee.

Rainbow trout (*Oncorhynchus mykiss*) purchased from Ocean Trout Farm Inc., Brookvale, PE were initially held in a 500-l tank with flow-through aerated well water at the Atlantic Veterinary College Aquatic Animal Facility. After acclimatization to laboratory conditions, the fish were divided into 2 groups of 60 fish and moved into two separate 250-l tanks. One group was maintained at 11 ^{o}C (cold acclimated; control) and the other acclimated to 20 °C (warm-acclimated) for 3 weeks. For warm acclimation, the temperature was increased gradually over 10 days to 20 ^{o}C and fish were maintained at this temperature for an additional 21 days. The

experimental temperatures I used are environmentally realistic for rainbow trout (Currie et al. 1998; Bear et al. 2007) and did not deviate significantly from the targeted levels over the acclimation period. Fish were fed daily at 1% body weight with commercial trout chow (Corey Feed Mills, Fredericton, NB).

5.3.2 Copper and hypoxia exposure

Following the 3 weeks of warm acclimation, fish from each group were divided into 4 treatment groups (Fig. 5.1) designated: control, Cu, hypoxia, and Cu plus hypoxia each with 6 fish (i.e., n = 6). The fish were then exposed to 20 µg/l Cu (as $CuSO_4$. $5H_2O$, Sigma-Aldrich Oakville, ON) and moderate hypoxia (45% saturation at each temperature) individually and in combination for 24 h at their respective acclimation temperature. There were two controls, one for each temperature with the fish at 11 ^oC being the overall experimental control. These static *in vivo* exposures were done in 150-l tanks and fish were not fed for a total of 48 h (24 h prior to moving to 150-l tanks + 24 h of experiment) before sampling. Due to logistical constraints, these exposures were done over 3 consecutive days. Temperatures in the exposure tanks were maintained using a chiller or immersion heater as appropriate. The desired level of hypoxia was achieved by balancing oxygen consumption by fish with bubbling of nitrogen-air mixture and maintaining a tight tank cover to minimize aerial breathing. Water quality parameters measured during the 24 h experimental period for 11 and 20 ^oC, respectively, were (n = 8-12), temperature: 10.9±0.48 and 20.1±0.59 ^oC; dissolved O_2: normoxic, 86.5±2.3 % saturation (9.43±0.36 mg/l) and 91.6±2.15 % saturation (8.40±0.25 mg/l); hypoxic, 46.5±2.5 % saturation (5.16±0.27 mg/l) and 46.6±2.5 % saturation (4.20±0.25 mg/l) and pH 7.60±0.14 and 7.94±0.24. The measured (atomic absorption spectrophotometry: PinAAcle 900T, Perkin Elmer, Woodbridge, ON) Cu concentrations were (µg/l), control: 0.73-1.12 and Cu exposures: 19.2-21.7 µg/l.

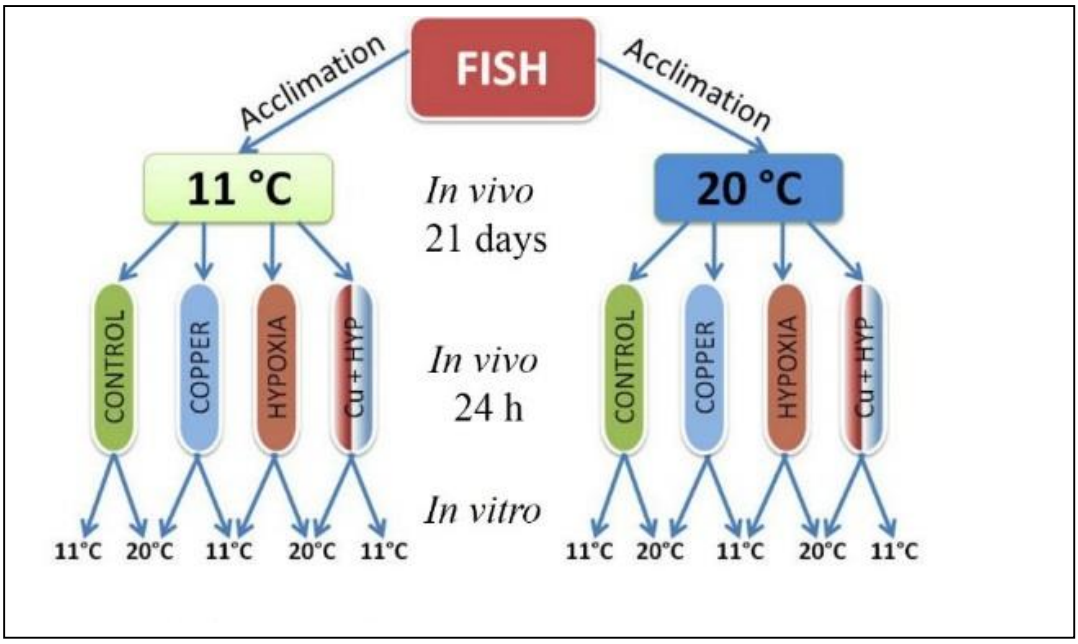


Figure 5.1: Experimental design for assessing effects and interactions of warm acclimation, acute temperature shifts, Cu and/or hypoxia on rainbow trout energy homeostasis. Acclimation, Cu and hypoxia exposures were done *in vivo* while acute temperature shifts were done *in vitro* with isolated liver mitochondria. Cu + HYP = copper plus hypoxia.

After the 24 h experimental period, fish were killed by a cephalic blow and weighed. Blood was collected using heparinized syringes by caudal venipuncture and submitted to in-house Atlantic Veterinary College Diagnostic Laboratory for glucose and lactate analysis. Fish were dissected to harvest livers, which were subsequently rinsed with ice-cold MIB (same composition as in chapter 2), blotted dry and weighed. The HSI was calculated as [wet liver mass/wet body mass] × 100.

5.3.3 Mitochondrial isolation and respirometry

Isolation of liver mitochondria followed my previously described protocol (Sappal et al. 2014a,b). Upon isolation, mitochondria were weighed and re-suspended in 3 volumes of EGTA-free MRB (same composition as in chapter 2) for respiration experiments. First, the protein concentrations of the mitochondrial suspensions were measured spectrophotometrically (Spectramax Plus 384, Molecular Device, Sunnyvale, CA) by the Bradford (1976) method. Respiration rates driven by mitochondrial ETS complexes I-IV (CI, CII, CIII and CIV) were measured with Clark-type oxygen electrodes (Qubit systems, Kingston, ON) housed in 4-ml cuvettes. A sequential inhibition-activation assay (Sappal et al. 2015a) allowed the measurement of respiration of all the four ETS complexes in one experimental run. Using this assay, the effect of acclimation (respiration rates measured at the acclimation temperatures of 11 or 20 °C) and acute temperature shift (cold shock: 20→11°C and heat shock: 11→20°C) on CI-CIV driven respiration rates were tested in controls and fish exposed to Cu, hypoxia and Cu plus hypoxia. The assay temperatures were maintained with a thermostatically controlled water circulator (Haake, Karlsruhe, Germany). Prior to all respiratory measurements, oxygen electrodes were calibrated at 0 and 100% air saturation, respectively, at ambient atmospheric pressure (740-760 mmHg) measured by a digital barometer (Fisher Scientific, Nepean, ON). The substrate-inhibitor combinations used were: CI: 5 mM malate and 5 mM glutamate, inhibitor not required; CII: 5 mM succinate with 0.5 µM rotenone, CI

inhibitor; CIII: 3 μM decylubiquinone (decylubiquinol reduced with potassium borohydride) with 25 μM malonate, CII inhibitor, and CIV: 5 mM ascorbate and 200 μM TMPD with 20 nM antimycin A, CIII inhibitor. Following measurement of CIV state 4, oligomycin (2.5 μg/ml), was added to inhibit CV in order to measure state 4_{ol}, an estimate of proton leak across the IMM (Brand and Nicholls, 2011). The details of this protocol have been presented in Sappal et al. (2015a).

All rates of O_2 consumption were recorded and analyzed using LabPro® software (Qubit Systems) and normalized to mitochondrial protein. The RCR and P/O ratios were calculated as described by Chance and Williams (1955) and Estabrook (1967).

5.4 Statistical analysis

The data are expressed as means ± SEM. Statistical analyses were performed with Statistica version 6.0 (StatSoft Inc., Tulsa, OK) with the level of significance set at $p < 0.05$. The data were initially tested for normality of distribution (Chi-Square test) and homogeneity of variances (Levene's test) and log transformed when necessary. The effects of temperature, Cu and hypoxia were analyzed via multivariate analysis of variance (MANOVA) followed by post-hoc pairwise comparisons of means using Tukey's HSD test. Condition indices were compared by *t* test while plasma metabolites were analyzed by one way ANOVA with Fisher's least significant difference (LSD) as the post hoc test.

5.5 Results

5.5.1 Fish condition and plasma metabolites profile

Fish acclimated to 20 ^{o}C had lower body (Fig. 5.2a) and liver (Fig. 5.2b) weights compared with fish maintained at 11 ^{o}C. Similarly, the HSI (Fig. 5.2c) was lower in the warm acclimated fish.

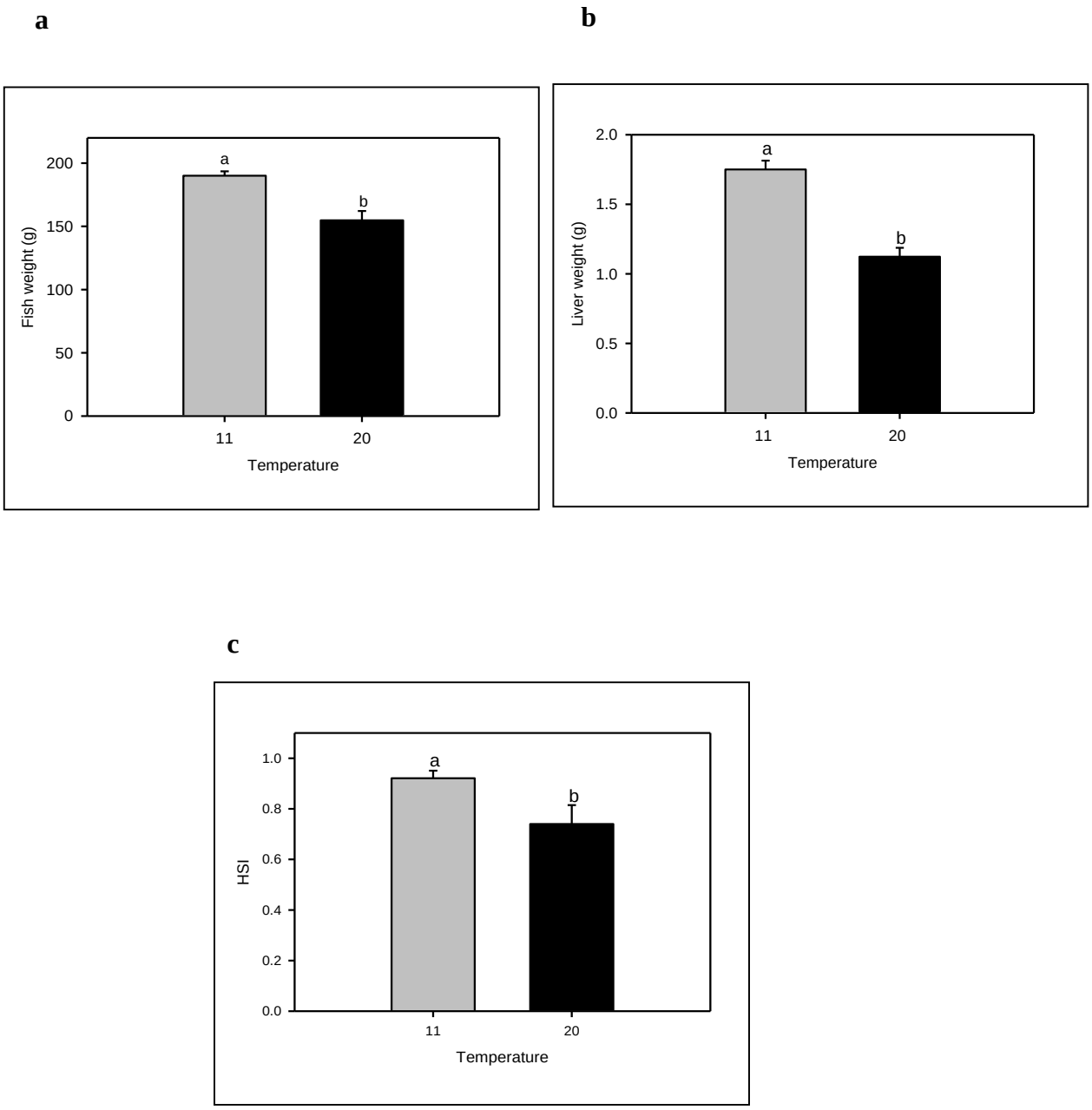


Figure 5.2: Effects of warm acclimation, Cu, hypoxia and Cu plus hypoxia on condition indices. Body weight (a), liver weight (b) and hepatosomatic index, HSI (c). Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, *t* test, $p < 0.05$.

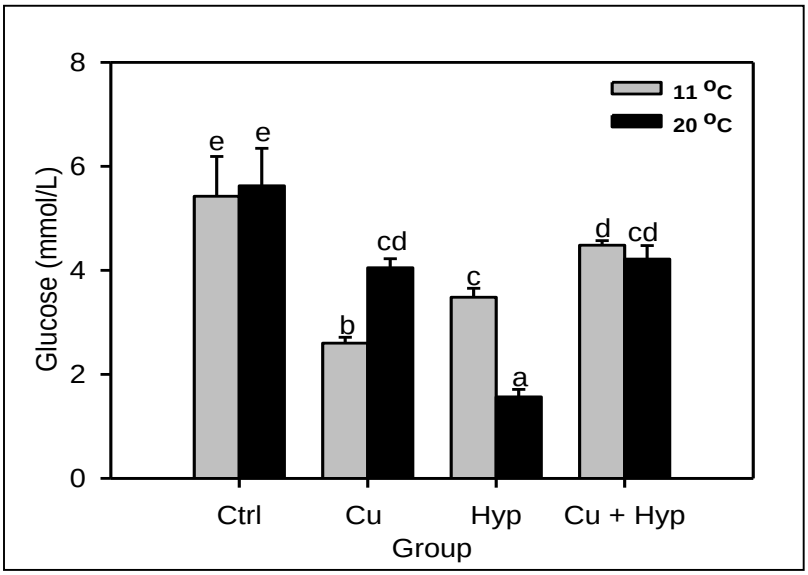
Plasma glucose concentrations (Fig. 5.3a) were not affected by warm acclimation but were reduced by Cu in both the cold and warm acclimated fish, albeit to a greater degree (52 vs. 28%) in the former. Hypoxia reduced plasma glucose concentration but in contrast with Cu, the effect was greater in the warm relative to the cold acclimated fish. In line with the opposing individual effects of Cu and hypoxia, Cu plus hypoxia reduced the glucose levels to the same degree in cold and warm acclimated fish but to a lesser extent than either stressor alone. Lactate levels on the other hand were 55% higher in warm relative to cold acclimated control fish (Fig. 5.3b). Exposure to Cu markedly reduced lactate levels in warm acclimated fish without affecting those of cold acclimated fish, whereas hypoxia reduced the lactate levels irrespective of the temperature acclimation status. Combined Cu and hypoxia reduced lactate in warm acclimated fish but did not alter it in cold acclimated fish.

5.5.2 Effects of warm acclimation, acute temperature shift, Cu, hypoxia and Cu plus hypoxia on ETS respiratory function

5.5.2.1 CI respiratory function

State 3 respiration for CI (Fig. 5.4a) was similar in warm and cold acclimated control fish when tested at the respective acclimation temperature despite the 9 ^{o}C temperature difference. In contrast, heat shock (11$\rightarrow$20 ^{o}C) increased CI state 3 by 66% whereas cold shock (20$\rightarrow$11 ^{o}C) decreased it by 142%. Cu exposure alone stimulated state 3 in both warm and cold acclimated fish mitochondria and alleviated the reduction in respiration imposed by cold shock. Hypoxia on the other hand had no effect on state 3 in both warm and cold acclimated control fish but it significantly reduced the stimulatory effect of heat shock. Lastly, while Cu plus hypoxia did not alter CI state 3 regardless of the acclimation status and direction of acute temperature change, it resulted in lower

a



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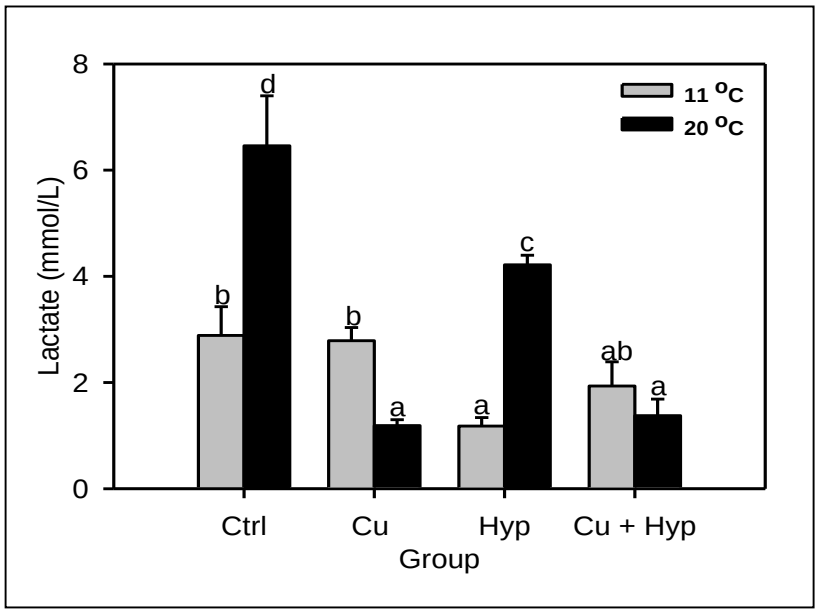

Figure 5.3: Effects of warm acclimation, Cu, hypoxia and Cu plus hypoxia on plasma metabolite profile. Glucose (a) and lactate (b). The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu + Hyp: 24 h 20 µg/l Cu + 24 h hypoxia. Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, LSD, $p < 0.05$.

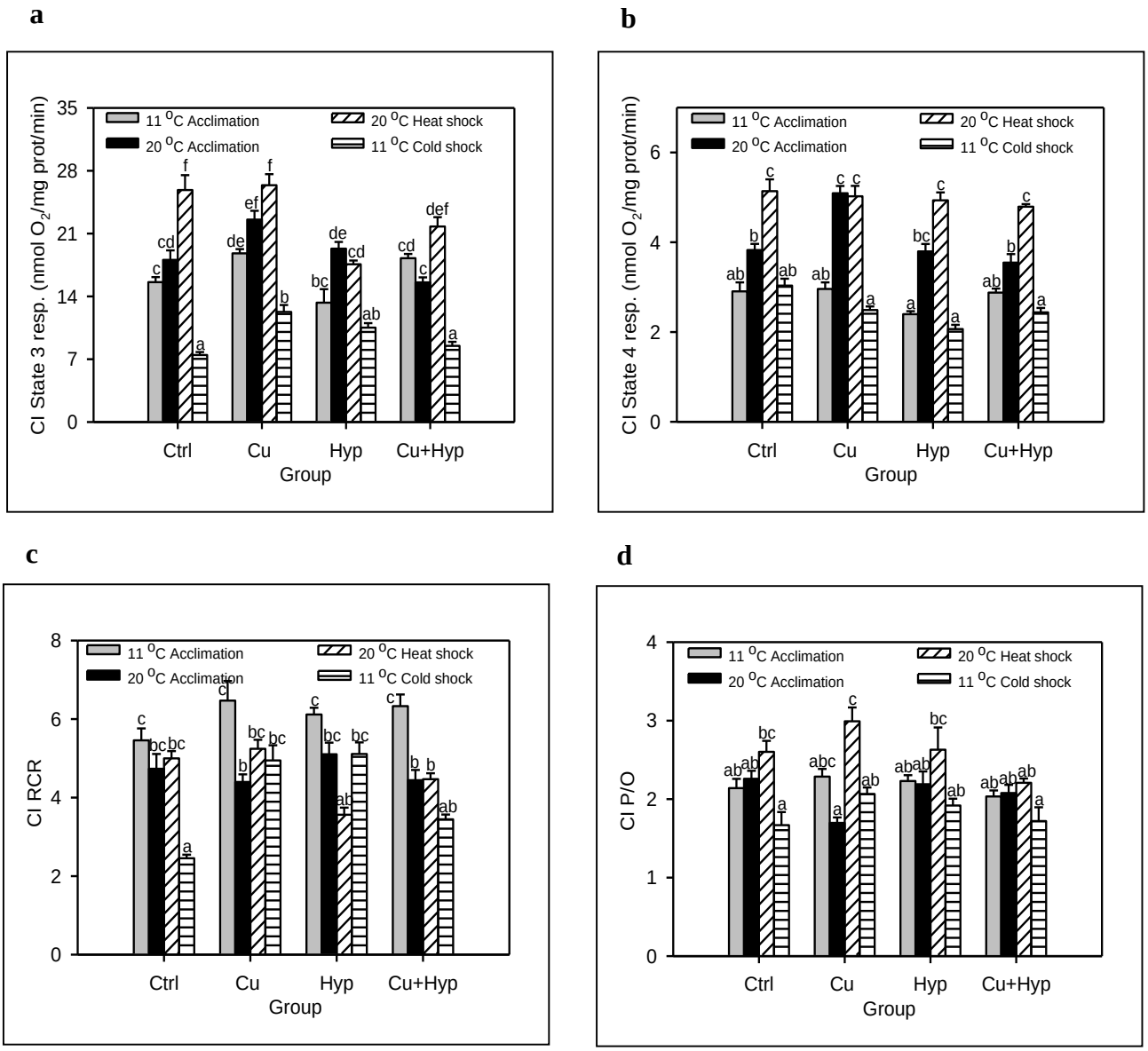

Figure 5.4: Effects of warm acclimation, acute temperature shifts, Cu, hypoxia, and Cu plus hypoxia on electron transport system complex I (CI) respiratory function. State 3 (a); state 4 (b); RCR (c); P/O ratio (d). The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu + Hyp: 24 h 20 µg/l Cu + 24 h hypoxia. Mitochondria isolated from each group were subjected to heat and cold shocks *in vitro*. Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, Tukey's HSD, $p < 0.05$.

respiration rates in warm acclimated fish and cold shocked mitochondria relative to Cu alone exposure.

CI-driven state 4 (Fig. 5.4b) in the controls was similar in cold and warm acclimated fish and was increased by heat shock (77%) but not significantly altered (26%) by cold shock. In contrast, Cu exposure increased state 4 in warm without altering it in cold acclimated fish mitochondria or after acute temperature shift. Hypoxia alone and in combination with Cu did not alter CI state 4 relative to the controls; however, it reduced the stimulatory effect of Cu in warm acclimated fish mitochondria.

The RCR for CI (Fig. 5.4c) was not affected by warm acclimation and heat shock in controls but was reduced (50%) by cold shock. Cu exposure did not alter CI RCR in cold and warm acclimated fish relative to the controls; however, it resulted in lower RCR in mitochondria from warm compared with cold acclimated fish. Interesting, Cu exposure reversed the cold shock evoked reduction in CI RCR while hypoxia reduced the RCR in heat shocked mitochondria and, similar to Cu, reversed the uncoupling effect of cold shock. Although in fish exposed to combined Cu and hypoxia CI RCRs were similar to the respective controls, this treatment resulted in higher RCR in mitochondria of cold acclimated fish relative to the other 3 groups, all of which were similar.

The phosphorylation efficiencies (P/O ratios) for CI (Fig. 5.4d) were similar in mitochondria from cold and warm acclimated control fish, and were not altered by heat or cold shock. While exposure to Cu and/or hypoxia did not significantly alter the P/O ratio in any group relative to the controls, warm acclimated fish mitochondria had lower P/O ratios than the cold acclimated after Cu exposure. Moreover, heat shock in Cu exposed fish mitochondria resulted in higher a P/O ratio than the other 3 groups. Lastly, while Cu and hypoxia jointly did not alter CI P/O ratio relative to

the respective controls or among the treatments within the group, it reduced the phosphorylation efficiency-enhancing effect of Cu in heat shocked mitochondria.

5.5.2.2 CII respiratory function

While state 3 for CII (Fig. 5.5a) was similar in cold and warm acclimated fish mitochondria, heat and cold shock increased and reduced it by 57 and 107%, respectively. Cu exposure increased CII state 3 relative to the respective controls in all except the heat-shocked group. In contrast, hypoxia abrogated the stimulatory effect of heat shock in cold acclimated fish mitochondria. Exposure to Cu and hypoxia jointly did not alter CII state 3 relative to the controls but it reduced the stimulatory effect of Cu in warm acclimated and heat shocked cold acclimated fish mitochondria.

The basal respiration for CII (Fig. 5.5b) was similar in cold and warm acclimation fish mitochondria, and was increased (74%) and reduced (69%) by heat and cold shock, respectively. Cu exposure in cold acclimated fish did not change CII state 4 but it stimulated it in warm acclimated fish. Hypoxia similarly did not significantly change CII state 4 irrespective of the acclimation status and acute temperature challenge, but it reduced it in heat shocked relative to the Cu exposed fish mitochondria. Combined Cu and hypoxia exposure did not modify CII state 4 relative to the controls but it increased this respiration rate in heat-shocked mitochondria relative to hypoxia exposure alone. Additionally, state 4 in warm acclimated fish mitochondria was lower following exposure to Cu plus hypoxia relative to either stressor individually.

The coupling efficiency (RCR) for CII (Fig. 5.5c) was reduced by warm acclimation and cold shock relative to the cold acclimated control. For this complex, the RCR was increased by Cu exposure in cold shocked mitochondria whereas hypoxia alone and in combination with Cu did

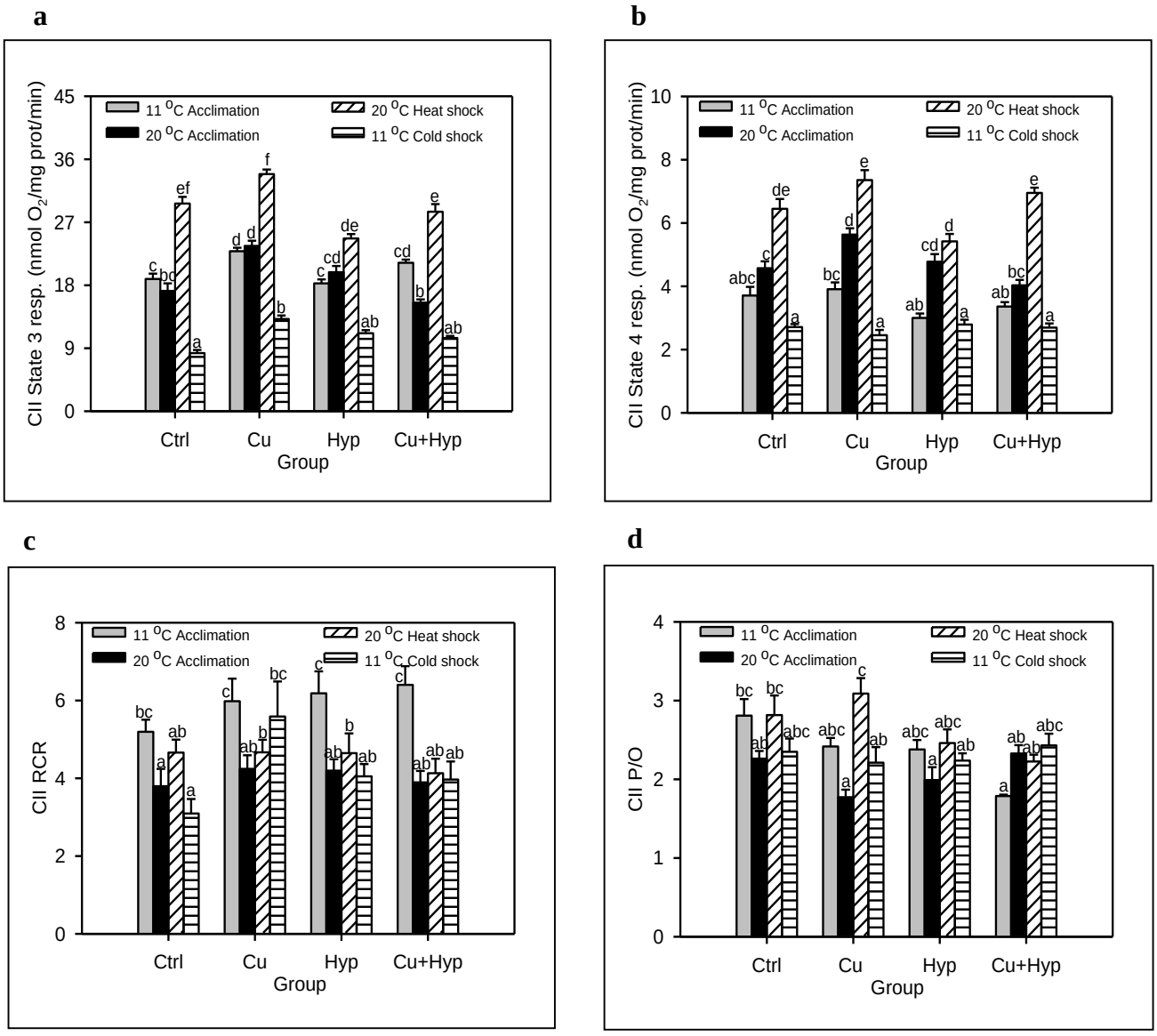

Figure 5.5: Effects of warm acclimation, acute temperature shifts, Cu, hypoxia, and Cu plus hypoxia on electron transport system complex II (CII) respiratory function. State 3 (a); state 4 (b); RCR (c); P/O ratio (d). The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu + Hyp: 24 h 20 µg/l Cu + 24 h hypoxia. Mitochondria isolated from each group were subjected to heat and cold shocks *in vitro*. Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, Tukey's HSD, $p < 0.05$.

not alter it relative to the controls. In both hypoxia and Cu plus hypoxia exposures, cold acclimated mitochondria had significantly higher RCR than the other three temperature combinations. For CII P/O ratio (Fig. 5.5d), thermal acclimation, acute temperature change and exposure to Cu and hypoxia all had no effect relative to their respective controls. However, Cu and hypoxia individually reduced the P/O ratio in warm acclimated fish mitochondria relative to the cold acclimated controls. Additionally, Cu plus hypoxia reduced CII P/O in cold acclimated fish mitochondria relative to the control as well as after heat shock in Cu exposed fish mitochondria.

5.5.2.3 CIII respiratory function

CIII state 3 (Fig. 5.6a) was similar in cold and warm acclimated control fish mitochondria. When mitochondria from cold acclimated fish were subjected to heat and cold shocks, CIII state 3 increased and decreased by 68 and 55%, respectively. Except for its reduction in heat shocked fish mitochondria by hypoxia alone and in combination with Cu, CIII state 3 remained comparable to the respective controls. Furthermore, hypoxia without and with Cu reduced the stimulatory effects of Cu in heat shocked mitochondria.

In contrast with state 3, CIII state 4 (Fig. 5.6b) was higher in warm relative to cold acclimated fish mitochondria. Heat and cold shocks increased and reduced CIII state 4 by 114 and 76%, respectively, while exposure to Cu had no effect relative to the controls. The effects of hypoxia alone and in combination with Cu were subtle with the only significant findings being that CIII state 4 in heat shocked mitochondria was inhibited by hypoxia while that of warm acclimated mitochondria was reduced by Cu plus hypoxia. The RCR for CIII (Fig. 5.6c) was reduced by warm acclimation but was not significantly altered by heat or cold shock. Cu exposure in contrast increased the RCR in cold acclimated and warm acclimated-cold shocked mitochondria.

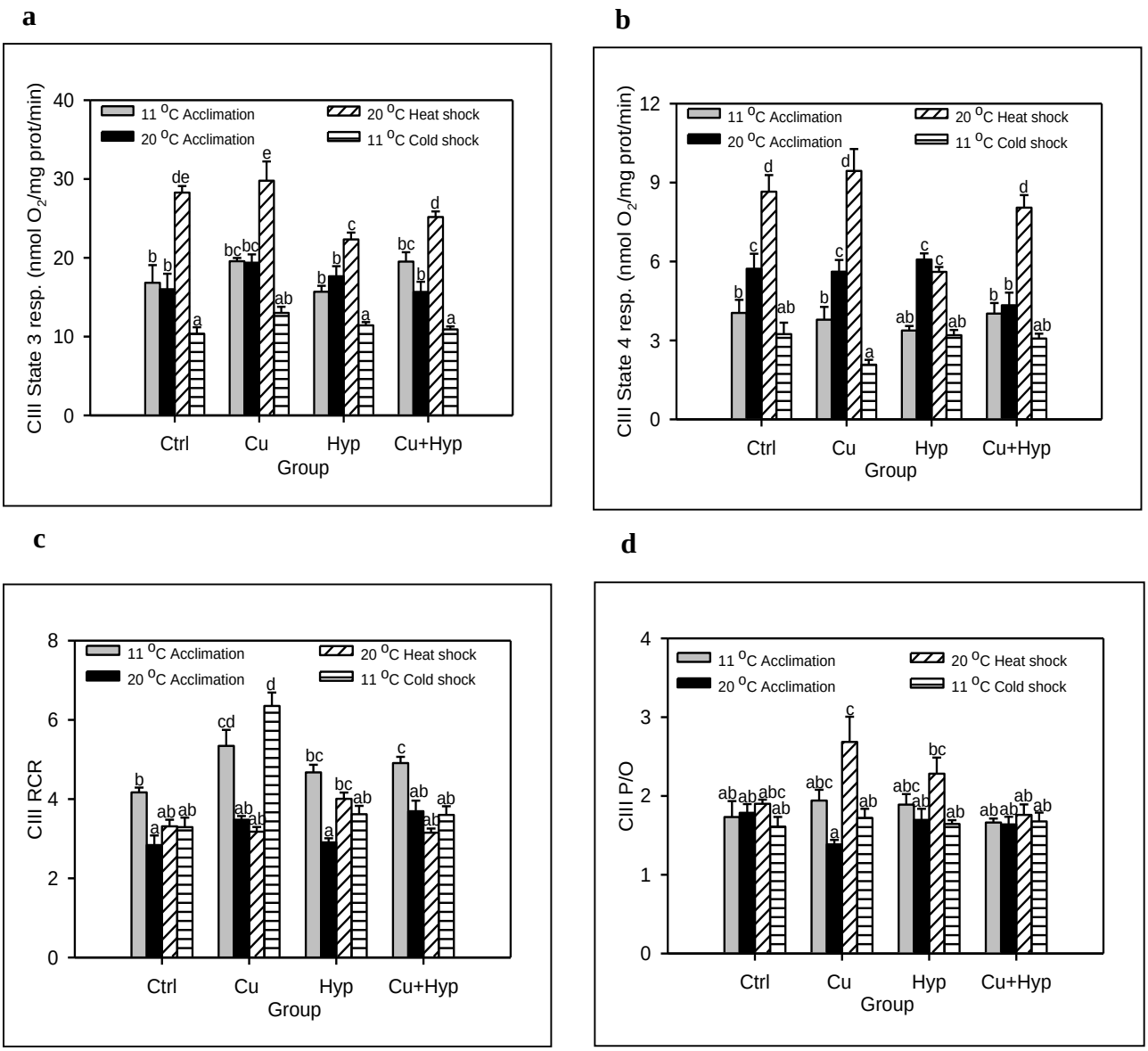

Figure 5.6: Effects of warm acclimation, acute temperature shifts, Cu, hypoxia, and Cu plus hypoxia on electron transport system complex III (CIII) respiratory function. State 3 (a); state 4 (b); RCR (c); P/O ratio (d). The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu + Hyp: 24 h 20 µg/l Cu + 24 h hypoxia. Mitochondria isolated from each group were subjected to heat and cold shocks *in vitro*. Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, Tukey's HSD, $p < 0.05$.

Except for the increase caused by Cu plus hypoxia in cold acclimated mitochondria, this treatment did not alter CIII RCR relative to the controls. Additionally, hypoxia without and with Cu reversed the RCR-enhancing effect of Cu in cold shocked mitochondria. For CIII P/O ratio (Fig. 5.6d), neither warm acclimation nor acute temperature change had significant effects in the controls. Similarly, exposure to Cu and hypoxia alone or in combination had no effect except that Cu increased P/O ratio in heat shocked mitochondria whereas Cu plus hypoxia reduced the stimulatory effect of Cu on this metric.

5.5.2.4 CIV respiratory function

CIV state 3 (Fig. 5.7a) was similar in controls and warm acclimated fish mitochondria; however, it was significantly increased (74%) and reduced (59%) by heat and cold shock, respectively. Cu, hypoxia and Cu plus hypoxia all reduced the stimulatory effect of heat shock on CIV state 3 while Cu plus hypoxia increased the inhibitory effect of Cu in heat shocked mitochondria. Similar to the effects on state 3, CIV state 4 (Fig. 5.7b) was comparable in cold and warm acclimated mitochondria and was increased by 94% and reduced by 59% following heat and cold shock, respectively. Exposure to Cu, hypoxia and Cu plus hypoxia all reduced the stimulatory effect of heat shock on CIV state 4. Hypoxia additionally stimulated state 4 of CIV in warm acclimated fish mitochondria with Cu plus hypoxia abolishing this effect. Cu plus hypoxia also inhibited CIV state 4 in heat shocked mitochondria to a greater extent than either stressor individually. State 4_{ol} (proton leak) of CIV (data not shown) was altered in exactly the same way as CIV state 4 albeit with, on average, 28% (20% in cold acclimated control) lower values. I found that warm acclimation and cold shock reduced CIV RCR (Fig. 5.7c) in the controls whereas Cu exposure without and with cold shock increased it in warm acclimated fish mitochondria. The effects of hypoxia alone and in combination with Cu were not significant.

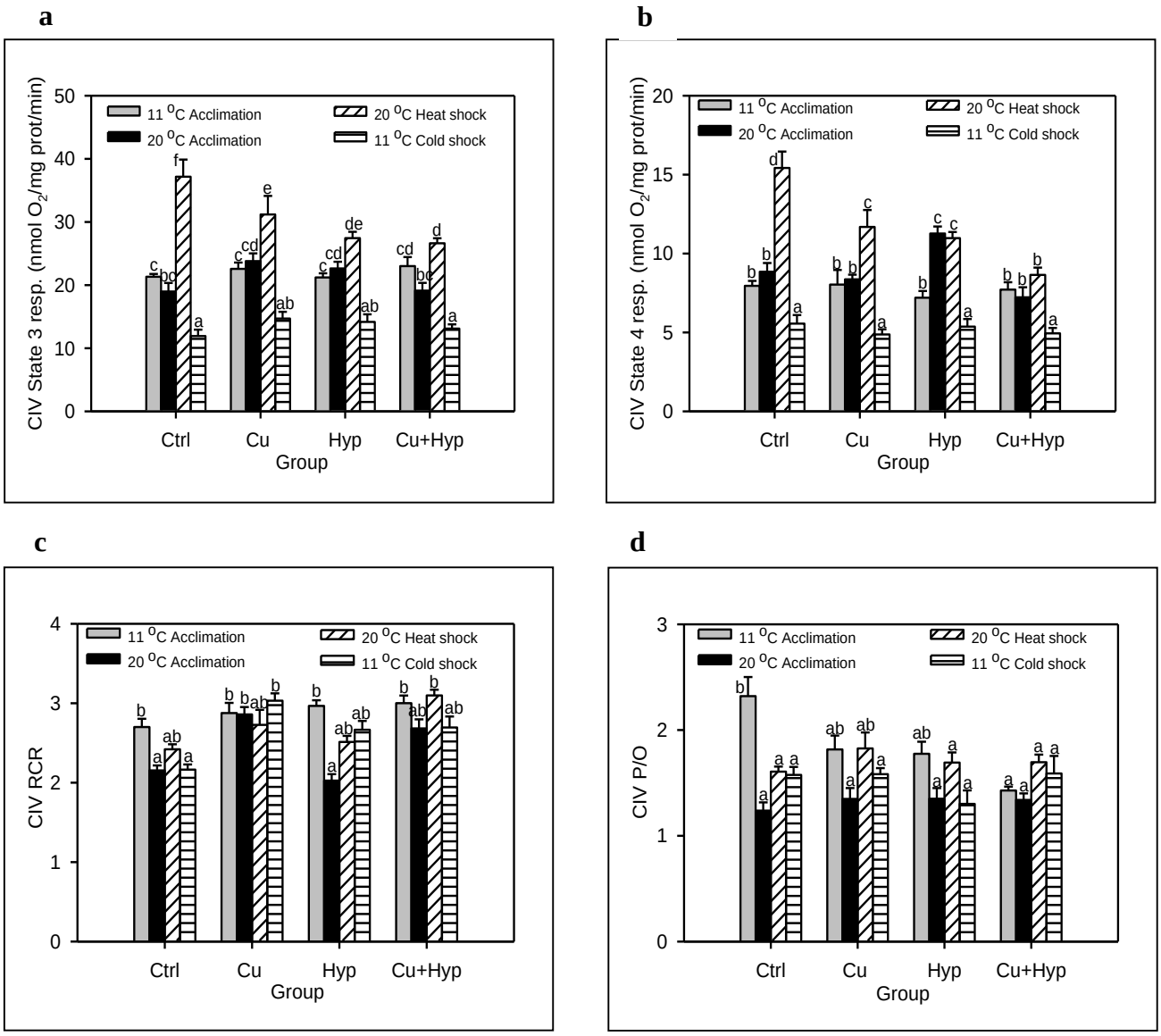

Figure 5.7: Effects of warm acclimation, acute temperature shifts, Cu, hypoxia, and Cu plus hypoxia on electron transport system complex IV (CIV) respiratory function. State 3 (a), state 4 (b), RCR (c), P/O ratio (d). The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu + Hyp: 24 h 20 µg/l Cu + 24 h hypoxia. Mitochondria isolated from each group were subjected to heat and cold shocks *in vitro*. Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, Tukey's HSD, $p < 0.05$.

Lastly, CIV P/O ratio (Fig. 5.7d) was reduced by warm acclimation and heat shock but was not altered by cold shock. Among the other treatments, only Cu plus hypoxia reduced CIV P/O in cold acclimated fish mitochondria.

5.5.3 Thermal sensitivities of CI-IV maximal and basal mitochondrial respiration rates

The thermal sensitivity (Q_{10} coefficient) of CI state 3 (Fig. 5.8a) in the controls was higher following acute drop (20→11 ºC) compared with acute rise (11→20 ºC) in temperature. Exposure to Cu and hypoxia singly and combined reduced CI thermal sensitivity for acute temperature drop whereas hypoxia without and with Cu reduced CI Q_{10} for acute temperature rise. The thermal sensitivity for CII state 3 showed a trend similar to that of CI, being higher following acute drop relative to acute rise in temperature (Fig. 5.8b). Here, only exposure to Cu plus hypoxia reduced the thermal sensitivity in cold shocked mitochondria. For CIII, state 3 Q_{10} values were similar irrespective of whether they were measured in warm acclimated fish mitochondria after acute temperature drop or in cold acclimated fish mitochondria after acute temperature rise. The only significant treatment-related change observed was that exposure to Cu plus hypoxia reduced the Q_{10} of acute temperature rise. Lastly, CIV state 3 Q_{10} values (Fig. 5.8d) were similar after acute rise and drop in temperature; however, exposure to hypoxia alone and in combination with Cu reduced the Q_{10} for acute temperature rise.

State 4 CI Q_{10} values (Fig. 5.9a) in the controls were lower when measured after acute drop relative to acute rise in temperature. Whereas Cu exposure resulted in higher Q_{10} values for acute temperature drop, hypoxia increased CI thermal sensitivity following either direction of the temperature change. For CII state 4 (Fig. 5.9b), the Q_{10} values in the controls were similar irrespective of whether they were calculated after acute rise or drop in temperature.

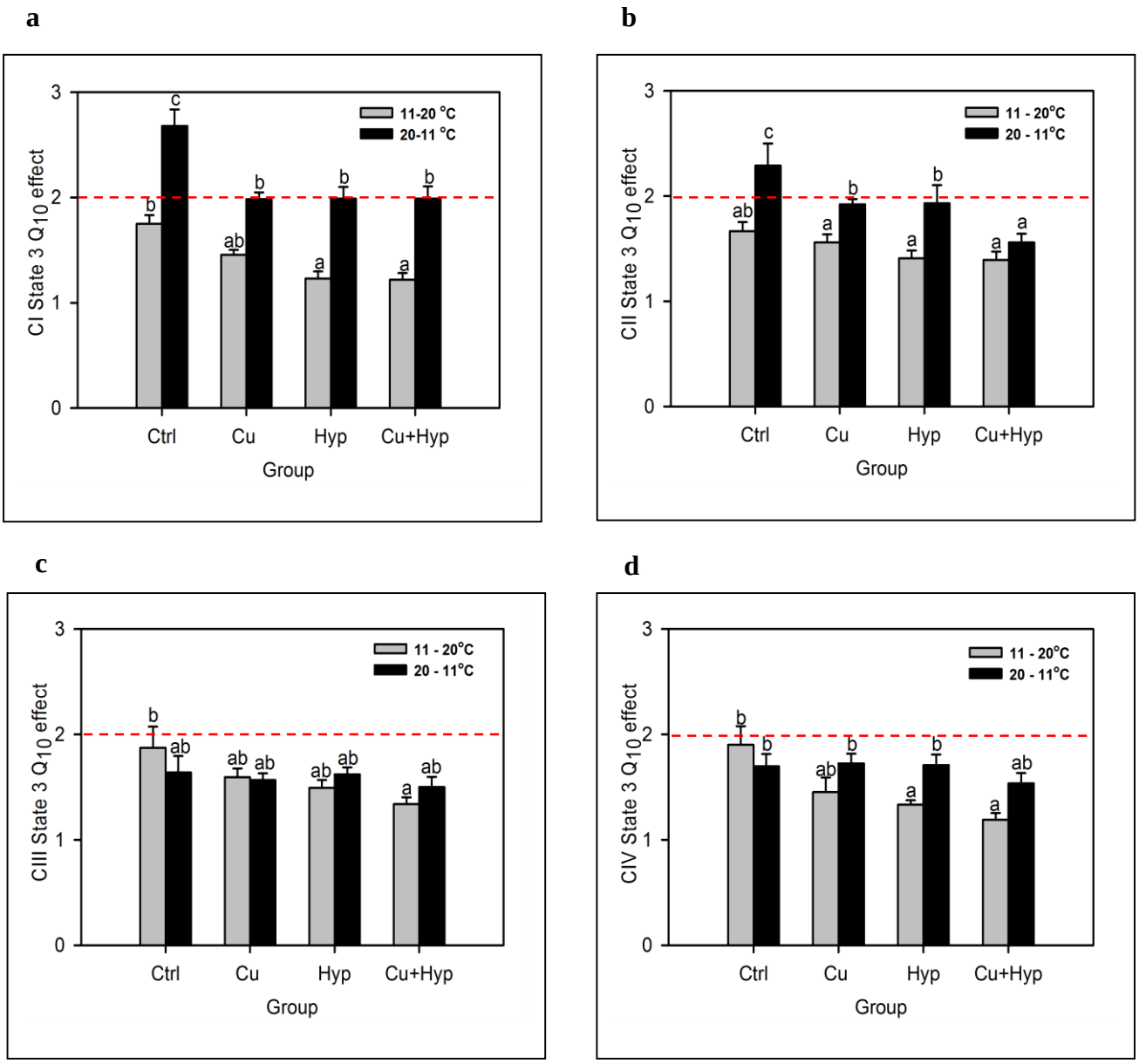

Figure 5.8: Effects of warm acclimation, acute temperature shifts, Cu, hypoxia, and Cu plus hypoxia on electron transport system complex I-IV (CI-IV) state 3 thermal sensitivity (Q_{10} coefficients). CI (a); CII (b); CIII (c); CIV (d). The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu + Hyp: 24 h 20 µg/l Cu + 24 h hypoxia. The red dashed line shows Q_{10} = 2. Mitochondria isolated from each group were subjected to heat and cold shocks *in vitro*. Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, Tukey's HSD, $p < 0.05$.

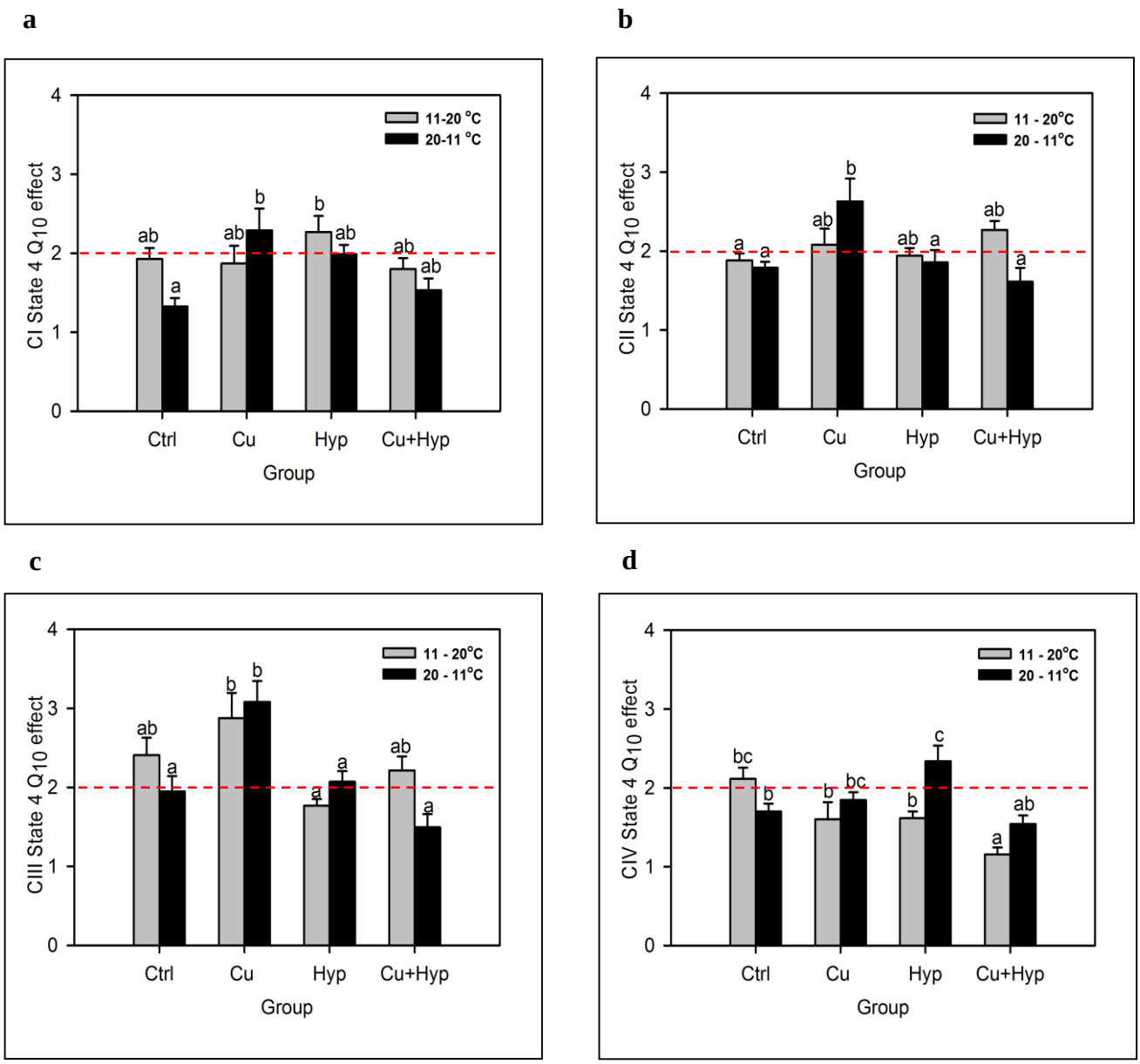

Figure 5.9: Effects of warm acclimation, acute temperature shifts, Cu, hypoxia, and Cu plus hypoxia on ETS complex I-IV (CI-IV) state 4 thermal sensitivities (Q_{10} coefficients). CI (a); CII (b); CIII (c); CIV (d). Experimental groups are: Ctrl: control; Cu: copper; Hyp: hypoxia; Cu + Hyp: Cu plus hypoxia. The red dashed line shows $Q_{10} = 2$. Mitochondria isolated from each group were subjected to heat and cold shocks *in vitro*. Values are means ± SEM, n = 6. Bars with different letters in each panel depict significantly different means, Tukey's HSD, $p < 0.05$.

Similar to CI, Cu exposure increased CII state 4 Q_{10} of acute temperature drop while hypoxia alone and in combination with Cu had no effect. Lastly, the thermal sensitivities for CIII (Fig. 5.9c) and CIV (Fig. 9d) state 4 were similar for acute rise and drop in temperature. Here, Cu exposure and hypoxia increased CIII and IV state 4 Q_{10} values measured after acute temperature drop, respectively, while Cu plus hypoxia reduced CIV Q_{10} after acute temperature rise.

5.6 Discussion

My study revealed that warm acclimation has profound effects on energy metabolism and alters how fish handle subsequent acute temperature shift, hypoxia and Cu stress. The mitochondrial functional impairment I observed was reflected in plasma metabolite profile and whole organism condition indices thus implicating modulation of mitochondrial bioenergetics in responses at higher levels of biological organization. While the temperature-hypoxia-Cu interactive effects were complex, they were mostly antagonistic with few additive actions.

5.6.1 Warm acclimation reduced fish condition

Condition indices are widely used in assessing fish fitness, health and nutritional status. In my study, fish acclimated to 20 ^{o}C had lower body and liver weights, as well as HSI, relative to the controls suggesting that warm acclimation reduced nutrient stores and/or capacity to transform nutrients to biomass. It is likely that my use of a relatively low feeding rate (1% body weight) coupled with increased metabolic demand at warm temperature contributed to the loss of condition. Similar to my findings, reduced growth and liver weights has been previously observed after warm acclimation (Bouchard and Guderley 2003; Grans et al. 2014; Sappal et al. 2015a) while cold acclimation increased liver/body mass (Kent et al. 1988; Seddon and Prosser 1997; Lannig et al. 2003).

5.6.2 Warm acclimation, Cu and hypoxia induced anaerobic metabolism

During stressful conditions high levels of cortisol and adrenaline promote glycogenolysis and gluconeogenesis in the liver to meet increased energy requirements, leading to elevated levels of glucose in plasma (Vijayan et al. 1997; Pankhurst 2010). Therefore in my study, the absence of change in plasma glucose levels after warm acclimation suggests that the temperature I tested did not present a significant stress to the fish or that fish made compensatory changes upon acclimation to the warm temperature. Alternatively, stress-induced increase in plasma glucose that may have occurred could have been matched by increased glucose oxidation. My findings, however, contrast an earlier study by Connors et al. (1978) that reported lower glucose levels in rainbow trout acclimated to 16 relative to 8 ^{o}C. Thus, differences in acclimation temperatures could explain, at least in part, the disparity with my findings.

My finding that Cu exposure reduced glucose levels agrees with a previous study in rainbow trout (Gagnon et al. 2006) and indicates that perhaps Cu increased glucose utilization and/or inhibited glucose replenishing pathways (e.g., gluconeogenesis). The glucose-depleting effect of Cu was less prominent in warm acclimated fish suggesting relatively greater stress and higher glucose mobilization at warm temperature. However, contrary to the common observation that hypoxia increases plasma glucose concentration due to mobilization of glycogen stores in the liver/muscle to fuel anaerobic glycolysis (Richards 2009) I found that this metabolite was reduced. It is possible that due to low nutrient (glycogen) stores as a result of the low ration I used, glycogenolysis induction during hypoxia could not match glucose requirement in tissues.

I found elevated plasma lactate in warm acclimated fish suggesting that persistent warm temperature stimulated anaerobic metabolism as recently reported for New Zealand wrasse,

Notolabrus celidotus (Iftikar et al. 2015). However, Connors et al. (1978) did not find changes in plasma lactate levels in rainbow trout albeit after acclimation to temperatures different from the one used in my study. It has been suggested that because glycolytic enzymes exhibit inverse or no thermal compensation, glycolytic metabolites occur at lower levels in cold relative to warm acclimated/adapted fish (Dunn and Johnston 1986; Guderley and Blier 1988). I did, however, find reduced plasma lactate levels in warm acclimated fish after Cu exposure which might be due to inhibition of lactate dehydrogenase because this enzyme is sensitive to Cu (Antognelli et al. 2003; Pamp et al. 2005), or increased lactate clearance in this group. Additionally, Lauer et al. (2012) reported both inhibition and stimulation of glycolytic enzymes by Cu in the estuarine crab (*Neohelice granulate*) that could result in reduced lactate levels if they occurred in my study. Previous studies in rainbow trout (Beaumont et al. 1995; Dethloff et al. 1999), however, did not report changes in plasma lactate suggesting that experimental conditions, e.g., the concentration of Cu used, may determine the outcome. Unexpectedly, I found that lactate was reduced in both warm and cold acclimated fish following hypoxia exposure. Although characteristics of anaerobic metabolism may fade over time if hypoxia is not deep enough, alternative explanations of my results include reduced availability of substrates for conversion to lactate and/or increased lactate clearance. Interestingly, Cu plus hypoxia prevented the lactate-enhancing effect of hypoxia in warm but not in cold acclimated fish, which offers additional support for the notion that Cu impairs glycolysis or promotes lactate clearance. Indeed, Speers-Roesch et al. (2010) reported a biphasic plasma lactate response to hypoxia in tilapia (*Oreochromis niloticus* × *mossambicus* × *hornorum*) wherein a decline in lactate after an early surge was attributed to its oxidation. Overall, my study agrees with the theme that plasma metabolites may not always follow a logical pattern (Haman et al. 1997) and suggests that they may not be robust measures of environmental stress.

5.6.3 Warm acclimation reduced maximal CI-IV mitochondrial respiration rates and altered effects of acute temperature change, hypoxia and Cu

In spite of the variability, the condition indices and plasma metabolite profile suggested that warm acclimation, hypoxia and Cu exposure altered energy metabolism. I hypothesized that modulation of mitochondrial function was fundamental to these changes and used my sequential inhibition-activation assay (Sappal et al. 2015a) to measure respiratory flux via all segments of the ETS to holistically characterize the effects and interactions of temperature, hypoxia and Cu. Overall, I showed that these stressors altered ETS respiratory activity differently and their interactions were mostly antagonistic.

Warm acclimation typically reduces mitochondrial oxidation capacity in fish (Bouchard and Guderley 2003; Lannig et al. 2005; Seebacher et al. 2010; Guderley and St-Pierre 2002; Guderley and Johnston 1996; Guderley 2011; Sappal et al. 2015a). Consistent with this theme the maximal oxidation rates for all the four ETS complexes were reduced following warm acclimation. This generalized reduction in respiration suggests that a core component of the mitochondria, e.g., membrane integrity (Hazel 1995; Guderley 2004; Guderley and St-Pierre 2002; Kraffe et al. 2007) was altered by thermal acclimation. Note that reduction of respiration with warm acclimation is beneficial because it abates oxygen requirement and ROS production at high temperature (Abele 2012).

My study revealed that warm acclimation altered how the ETS responded to acute temperature shift and Cu and/or hypoxia stresses. First, the effect of acute temperature change depended on the ETS complex and acclimation status. Specifically, CI and II in warm acclimated fish mitochondria exhibited greater thermal sensitivities relative to the cold acclimated whereas CIII and IV thermal

sensitivities were similar irrespective of the acclimation status. This proximal (CI and II) versus distal (CIII and IV) dichotomy suggests that acute temperature change modulated these ETS proteins differently. Second, Cu exposure stimulated CI and CII state 3 consistent with my recent findings on effects of low doses of Cu *in vitro* (Sappal et al. 2014 a,b, 2015a). The mechanisms of the stimulatory effect of Cu on CI and II maximal respiration remain unknown but they might involve changes in conformation and activity of the two enzymes. Indeed, CI was reported to exist in an active and de-active conformation, the relative proportion of which is altered by chemicals and hypoxia (Galkin et al. 2009). On the other hand, Cu^{2+} might directly or by altering expression of genes and mimicry of divalent cations such as Ca^{2+} and Mg^{2+}, activate enzymes including those of the ETS. Furthermore, the lessening of the inhibitory effect of acute temperature drop in CI, II and CIV by Cu, suggests that this metal might have a stabilizing effect on mitochondria that enhances the resistance of these organelles to acute temperature challenge. Overall, while my study revealed potentially beneficial effects of Cu, high levels of this metal both *in vitro* (Sappal et al. 2014a,b; Belyaeva et al. 2011) and *in vivo* (Maes et al. 2013; Ransberry et al. 2015) clearly inhibit the ETS.

Hypoxia typically impairs maximal mitochondrial oxidation rates (Heerlein et al. 2005; Richards 2011; Solaini et al. 2010). In my study, the level of hypoxia tested reduced the stimulatory effect of acute temperature rise on CI, III and IV state 3 indicating that this stressor acts via mechanisms antagonistic to those of temperature. Because hypoxia did not alter the effect of cold shock, warm acclimation arguably could have increased hypoxia tolerance as previously reported (Portner 2010; Burleson and Silva 2011; Anttila et al. 2015). Alternatively, acute temperature rise is probably more stressful than acute drop because it accelerates cellular metabolism thus increasing oxygen demand while concurrently reducing the oxygen content of the water. Lastly, the subtle effects of

Cu plus hypoxia on state 3 is strikingly different from my recent *in vitro* study in which Cu combined with HRO caused clearly greater inhibition of CI-IV than either stressor alone (Sappal et al. 2015a). HRO is nonetheless different from hypoxia, and the levels of Cu and hypoxia I tested here *in vivo* were lower.

5.6.4 Warm acclimation reduced basal mitochondrial respiration and altered the effects and interactions of acute temperature shift, hypoxia and Cu

Warm acclimation reduced basal mitochondrial respiration consistent with previous studies on different ETS complexes (Guderley and Johnston 1996; Kraffe et al. 2007; Sappal et al. 2015a). My study is also in agreement with earlier findings that state 4 (proton leak) increases with acute temperature rise (Hardewig et al. 1999a; Fangue et al. 2009; Sappal et al. 2014a,b). It appears that warm acclimation and acute temperature change altered IMM permeability (Guderley 2004; Guderley and St-Pierre 2002; Kraffe et al. 2007) and/or modulated the mechanisms (Murphy 2009; Jastroch et al. 2010; dos Santos et al. 2012) that mediate proton leak. Contrasting the generalized effect of temperature, the effects of Cu exposure on basal respiration were restricted mainly to CI and II, lending additional support to the notion that Cu acted specifically on ETS proteins. Moreover, the stimulation of CI and CII state 4 observed here is consistent with Sappal et al. (2014a,b) and shows concordance between *in vitro* and *in vivo* responses. Interestingly similar to state 3, I observed a proximal-distal dichotomy in the sensitivity of state 4 to hypoxia, and reciprocally antagonistic effects between hypoxia and Cu.

5.6.5 RCR was more responsive to warm acclimation, acute temperature shift, hypoxia and Cu than the P/O ratio

Warm acclimation consistently reduced the RCR for all the ETS complexes and sensitized the mitochondria to acute temperature-induced uncoupling. Similar to my findings, others (Bouchard and Guderley 2003; Iftikar et al. 2015) reported reduced ETS coupling efficiency following warm acclimation. Clearly, warm acclimation comes at a cost of reduced efficiency of the ETS. In my study, warm acclimation and cold shock reduced RCR primarily by inhibiting state 3 while heat shock induced uncoupling by stimulating state 4. Interestingly, Cu exposure reversed cold shock imposed reduction of RCR for all of the ETS complexes except CIV while its effects in cold acclimated mitochondria were restricted to enhancement of CIII RCR. The mechanisms underlying this apparent improvement of coupling by Cu under cold conditions remain to be determined but my data do show that at low temperature Cu stimulated state 3 more than it did state 4. In contrast, effects of hypoxia were limited to CI where it reduced RCR in heat shocked mitochondria and reversed the uncoupling effect of cold shock. As indicated earlier, the sensitivity of CI to hypoxia is likely due to the phenomenon of active-to-deactive form transition. Moreover, the finding that Cu plus hypoxia did not alter CI-IV coupling indicates antagonistic interaction when the stressors were combined.

The phosphorylation efficiency was less responsive to temperature than the RCR but it was reduced by warm acclimation (CIV) and heat shock (CIII and IV). Previous studies have reported reduced (Dufour et al. 1996; Bouchard and Guderley 2003; Lemieux et al. 2010; Onukwufor et al. 2015), increased (Lemieux et al. 2010; Sappal et al. 2014b) and unchanged (Guderley and Johnston 1996; Hardewig et al. 1999a; Portner et al. 1999a; Bouchard and Guderley 2003; Lannig et al. 2005; Rodnick et al. 2014) P/O ratios for a variety of ETS complexes following warm acclimation

or acute temperature increase. This variability in P/O ratio can be explained to a large extent by differences in acclimation and/or assay temperatures. Similarly, the effects of Cu and/or hypoxia on P/O ratio were variable with Cu generally increasing (CI and CIII) and hypoxia without and with Cu reducing (CI-CIII) the ratios under various states of thermal stress. While studies on joint effects of hypoxia and Cu on P/O ratio are limited, for hypoxia alone, unchanged (Kurochkin et al. 2009), reduced (Hoffman et al. 2007; Onukwufor et al. 2014) and increased (Gnaiger et al. 2000; Sussarellu et al. 2013) ratios have been reported. Taken together, the variability of alterations in P/O ratio suggests that this metric is not a persuasive test for the impact of stressors on ETS.

5.6.6 ETS thermal sensitivity displayed anterior-distal dichotomy with inverse responses of state 3 versus state 4 to temperature, hypoxia and Cu

In my study, the anterior ETS segments exhibited higher state 3 temperature coefficients in warm compared with cold acclimated fish mitochondria whereas in the distal segments, thermal sensitivities were similar irrespective of the acclimation status. These findings support the idea that thermal sensitivities of enzymes of the ETS are different (Fangue et al. 2009; Lemieux et al. 2010; Pichaud et al. 2010; Oellermann et al. 2012). However, in stark contrast with state 3, state 4 (proton leak) of the posterior segments was more sensitive to temperature rise than the anterior segments. These diametrically opposite thermal sensitivities for state 3 and 4 indicate that loss of efficiency in CIII and IV may be counteracted by increased efficiency in CI and II, and underscores the need to assess effects of stressors on multiple ETS segments.

Overall, the key findings of my study regarding thermal sensitivities of maximal and basal respiration rates driven by ETS CI-IV were: first, warm acclimation increased thermal sensitivities of state 3 but reduced those of state 4. Second, Cu and hypoxia individually either did not alter or

increased the thermal sensitivities of state 4 particularly in warm acclimated fish mitochondria, and either did not alter or reduced those of state 3. Third, Cu plus hypoxia either reduced or did not alter state 3 thermal sensitivities, and either did not alter or reduced the stimulatory effect of Cu or hypoxia on state 4 thermal sensitivities. While these varied effects imply that stressors acted differently to modulate ETS responsiveness to temperature, the antagonistic effects suggest the existence of some shared mechanisms. Moreover, a potential consequence of different thermal sensitivities among ETS complexes, along with the fact that they are modulated differently by Cu and/or hypoxia, is that fish could recruit appropriate ETS segments to sustain energy homeostasis depending on the stressors they encounter. For example, Oellermann et al. (2012) argued that mitochondrial segments that have low thermal sensitivity lessen the reduction of ATP production at low temperature whereas those with high sensitivity supplement the less responsive segments.

5.7 Conclusions

Warm acclimation reduced fish condition and altered plasma metabolite profile in a manner indicative of increased stress and stimulated anaerobic metabolism. The changes imposed by warm acclimation altered how fish responded to acute temperature shifts, hypoxia and Cu stress. Importantly, changes at organism and tissue levels could be explained to a significant extent by changes in mitochondria function underscoring importance of mitochondrial mechanisms in mediating the apical effects of these stressors. For the most part, warm acclimation and hypoxia reduced while Cu, particularly after cold shock, increased mitochondrial coupling efficiency. Moreover, Cu and hypoxia showed reciprocal antagonistic interactions with a few additive effects limited to state 4 in the distal ETS complexes. Overall, my study revealed previously unknown joint effects of temperature, hypoxia and Cu that underscore the importance of assessing multiple ETS complexes for a more holistic perspective of mitochondrial function.

CHAPTER 6

TRANSCRIPTIONAL AND FUNCTIONAL IMPACTS OF MULTIPLE STRESSORS ON ENERGY METABOLISM AND STRESS RESPONSE IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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6.1 Abstract

To survive in changing environments fish utilize wide range of biological responses, sustenance of which requires energy. I examined how warm acclimation influences the ETS enzymes and select transcriptional responses to hypoxia and Cu exposure in fish. Rainbow trout (*Oncorhynchus mykiss*) were acclimated to cold (11 ^{o}C; control) and warm (20 ^{o}C) temperature for 3 weeks followed by exposure to Cu, hypoxia or both for 24 h. Activities of ETS enzyme complexes I-IV (CI-CIV) were measured in liver and gill mitochondria. Analyses of transcripts encoding for proteins involved in mitochondrial respiration (cytochrome c oxidase subunits 4-1 and 2: COX4-1 and COX4-2), metal detoxification/stress response (metallothioneins A and B: MT-A and MT-B) and energy metabolism/sensing (AMP-activated protein kinase α1: AMPKα1) were done in isolated liver mitochondria, whole liver and gill tissues by RT-qPCR. Warm acclimation inhibited activities of ETS enzymes while effects of Cu and hypoxia were variable depending on enzyme and thermal acclimation status. The genes encoding for COX4-1, COX4-2, MT-A, MT-B and AMPKα1 were strongly regulated by warm acclimation being either upregulated or downregulated depending on tissue. While Cu and hypoxia clearly increased MT-A and MT-B transcript levels in all tissues, their effects on COX4-1, COX4-2 and AMPKα1 mRNA levels were less pronounced. Interestingly, warm acclimation increased and reduced COX4-2/COX4-1 in liver mitochondria and gill tissue, respectively. I unveiled both independent and joint actions (antagonism and potentiation) of 3 stressors on ETS enzymes and transcribed genes involved in energy metabolism, stress response and metals homeostasis. Overall, my study not only provided the mechanistic underpinnings of responses of fish to thermal stress, hypoxia and Cu, but unveiled novel interactive effects that should not be overlooked in real world situations wherein fish normally encounter multiple stress factors.

6.2 Introduction

The physical and chemical properties of many aquatic environments fluctuate due to natural and anthropogenic processes. While aquatic organisms utilize a variety of strategies ranging from behavioral to the molecular level to survive in changing environments, a common denominator in their repertoire of responses to environmental change is the modulation of energy metabolism (Sokolova et al. 2012). As a corollary, mitochondria, the organelles that generate the majority of the cellular energy (ATP), occupy a critical locus in the response to environmental change. Mitochondria produce ATP via OXPHOS, which is accomplished by the ETS, a set of enzymes associated with the IMM. In this multistep process, electron transport is coupled with pumping of protons from the matrix across the IMM into the intermembranous space, generating a pmf that is used by the CV to synthesize ATP. Thus, alterations in this process could be a sensitive indicator of stress-induced disruption of energy balance (Guderley and St-Pierre 2002; Galli et al. 2013; Blier et al. 2014) and by extension cellular/organismal functional integrity. Moreover, fish typically encounter a melange of potential stressors in their natural environment resulting in joint actions that may alter biological outcomes relative to individual stressors. While recent studies (Antilla et al. 2015; Sappal et al. 2015a,b) demonstrated the existence of additive, synergistic and antagonistic interactions, it is unclear how the fundamental cellular energy generating and sensing mechanisms respond to multiple stressors. Among the numerous potential stressors, fluctuations in temperature and oxygen levels, and the presence of chemical contaminants are arguably the most consequential in aquatic systems.

Temperature is a highly influential environmental variable particularly in ectothermic species such as fish because the temperature of the internal milieu of most fish, and thus rates of biological processes, changes directly with that of the external environment. Both acute and

persistent temperature changes induce stress and alter energy metabolism (Guderley and St-Pierre 2002; Kraffe et al. 2007; Blier et al. 2014). Specifically, acute temperature rise or drop induces corresponding changes in mitochondrial oxidation rates while long term temperature shifts induce compensatory changes via the phenomenon of acclimation (Guderley and St-Pierre 2002; Hochachka and Somero 2002; Guderley and Johnston 1996). Acclimatory modifications include but are not limited to changes in mitochondrial content and structure as well as alteration in the function of proteins that mediate energy metabolism (Kraffe et al. 2007; St-Pierre et al 1998; Bouchard and Guderley 2003). Therefore measuring the activity and expression of the biochemical machinery that regulates energy balance may shed light on the mechanisms underlying the energetic impact of thermal stress and how it affects the handling of other stressors.

Hypoxia in many aquatic environments is typically a diurnal or seasonal phenomenon driven by imbalance between the oxygen generating (photosynthesis) and consuming (e.g., respiration by organisms, biochemical oxygen demand) processes. In recent years, the severity and incidence of hypoxia has been aggravated by eutrophication and global warming (Diaz and Rosenberg 2008) and as a result, the combination of environmental fluctuations in temperature and oxygen levels presents a significant and worsening threat in many aquatic systems. Fish respond to hypoxia via mechanisms that either increase the uptake and/or reduce the consumption of oxygen while enhancing oxygen-independent energy generation (Richards 2009). Thus, there is a convergence of effects of temperature and hypoxia on energy metabolism that suggests that interactive responses are possible and could confound interpretation/prediction of the impacts of these stressors in fish. Indeed, temperature tolerance in fish is altered by hypoxia and vice versa

(Portner and Lannig 2009; Burleson and Silva 2011; McBryan et al. 2013) but much remains unknown regarding the mechanisms underlying the interactions of these stressors.

The effects of temperature stress and hypoxia in fish could be further cofounded by pollution of the aquatic system by metals. While the energetic cost of metals exposure in aquatic organisms has not been comprehensively investigated, metals can disrupt energy balance by directly impairing mitochondrial function or increasing basal metabolism (Sokolova et al. 2012; Sappal et al. 2014a,b, 2015a,b). Cu is a trace metal of concern because it is commonly found at elevated levels in aquatic systems following its release by natural processes or anthropogenic activities. Although essential, at high concentrations Cu is toxic due to its ability to catalyze the generation of ROS and propensity to bind ectopically to proteins disrupting their structure and function (Valko et al. 2005; Stohs and Bagchi 1995). Therefore cellular levels of Cu are strictly controlled via a homeostatic system that regulates its uptake, distribution, excretion and sequestration (Kamunde and Wood 2004; Bury et al. 2003; Balamurugan and Schaffner 2006). Sequestration, which reduces the free Cu ion levels and potential for ectopic binding, is mainly performed by MTs (Roesijadi 1996; Amiard et al. 2006; Balamurugan and Schaffner 2006), low molecular weight (6-7 kDa) cysteine-rich proteins. Two isoforms of MT, tMT-A and tMT-B, have been described in rainbow trout (Bonham et al. 1987; Zafarullah et al. 1988) and up-regulation of their transcription has been detected in several tissues following exposure to metals (Van Cleef-Toedt et al. 2001; Van Heerden et al. 2004). However, because MTs can be induced by stressors other than metals (Ivanina et al. 2009; Pierron et al. 2007; Amiard et al. 2006), it is important to understand the role of these proteins during exposure to multiple stressors.

The goal of the present study was to unveil the biochemical and transcriptional mechanisms that underlie responses to temperature, hypoxia and Cu stresses in fish. I focussed on mechanisms

involved in energy generation, metal homeostasis and stress response. First, I measured the activities of ETS complexes I to IV (CI-IV) to test the prediction that alterations in OXPHOS observed in my recent study (Sappal et al. 2015b) resulted from changes in enzyme activities. Second, the expression of select genes encoding proteins involved in metals sequestration, stress response and energy metabolism were assessed to determine the potential mechanisms of temperature-hypoxia-Cu interactions. Moreover, I conducted these analyses on liver and gill samples to assess the tissue specificity of the responses. Overall, my study identified the individual and joint responses induced by temperature, hypoxia and Cu stress in fish with implications for the prediction of effects of multiple stressors.

6.3 Material and methods

6.3.1 Fish and experimental procedures

All experimental procedures for the use of fish followed the Canadian Council on Animal Care guidelines as approved by the University of Prince Edward Island Animal Care Committee (protocol #11-034). The procedures including temperature acclimation, exposure to hypoxia and Cu and isolation of liver mitochondria have been detailed in my companion publication (Sappal et al. 2015b). Here I highlight the salient experimental issues and describe additional procedures specific to the present study. Briefly, rainbow trout (*Oncorhynchus mykiss*) were acclimated to 11 ^{o}C (cold acclimated; control) and 20 °C (warm-acclimated) for 3 weeks. The fish were then exposed to 20 μg/l Cu (as $CuSO_4$ $5H_2O$, Sigma-Aldrich Oakville, ON) and hypoxia (45% saturation) alone and in combination for 24 h at the respective acclimation temperatures. Livers and gills from 6 fish per experimental group (i.e., n = 6) were harvested and used as sources of mitochondria for measurement of ETS enzymes' activities, and whole tissue homogenates for

gene expression. These samples were snap frozen using dry ice during sampling and stored at -80 °C until further processing.

6.3.2 Isolation of hepatic and gill mitochondria for biochemical and gene expression analyses

Isolation of liver mitochondria followed my previously described procedure (Sappal et al. 2015a,b). The isolation of gill mitochondria followed a similar procedure as for the liver with the following modifications: (i) the isolation buffer (250 mM sucrose, 8 mM EDTA, 10 mM HEPES, 1 mg/ml BSA (fatty acid free) and 2 µg/ml aprotonin, pH 7.0) was used at 1:5 (wt/vol) ratio, (ii) due to low mitochondrial yield, the first 800 × *g* pellet was taken through a second mitochondrial isolation procedure and two mitochondrial subsamples were pooled, (iii) the mitochondrial resuspension buffer contained 200 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 10 mM HEPES, 1 mg/ml BSA (fatty acid free) and 2 µg/ml aprotonin, pH 7.0.

6.3.3 Measurements of activities of ETS enzyme complexes in liver and gill mitochondria

The protein concentrations of liver and gill mitochondrial suspensions were initially measured spectrophotometrically (Spectramax Plus 384, Molecular Device, Sunnyvale, CA) by the Bradford (1976) method. Thereafter, activities of all four enzyme complexes of the ETS (CI-IV) were measured in both liver and gill mitochondria for all exposures. Using liver and gills enabled us to evaluate tissue specificity of the responses.

6.3.3.1 Complex I (NADH:ubiquinone oxidoreductase) activity

CI activity was measured according to Sappal et al. (2014a) as modified from Spinazzi et al. (2012). Briefly, mitochondria were diluted to 6 mg/ml in hypotonic buffer (25 mM potassium phosphate and 5 mM $MgCl_2$) and sonicated to disrupt the IMM thus liberating the membrane

bound enzyme. Subsequently, 240 µl of assay buffer (25 mM of potassium phosphate, 3.5 g/l of BSA, 0.1 mM DCPIP, 280 µM decylubiquinone, 0.6 µg/ml antimycin A and 0.2 mM NADH, pH 7.3) and 60 µg of mitochondrial protein were added to wells of a 96-well microplate. The assays were done in triplicates for each temperature-Cu and/or hypoxia combination with the changes in absorbance being read at 600 nm for 5 min at 15 s intervals (Spectramax Plus 384) without and with addition of 3 µl of 1 mM rotenone which blocks CI activity. CI activity was then calculated by subtracting the rotenone-insensitive activity from the total activity.

6.3.3.2 Complex II (succinate:ubiquinone oxidoreductase) activity

CII activity was measured according to the method of Sappal et al. (2014b) as modified from Spinazzi et al. (2012). In this redox assay, succinate was the donor, while DCPIP was the acceptor of electrons. First, the mitochondrial suspensions were diluted to 6 mg/ml in hypotonic buffer and sonicated to disrupt the IMM. Thereafter, 60 µg of mitochondrial protein (10 µl) was added to 240 µl of assay buffer (pH 7.3) containing 25 mM potassium phosphate, 18 mM sodium succinate, 0.1 mM DCPIP, 2.5 mM KCN, 2.5 µg/ml antimycin A, and 10 µM rotenone. Absorbance at 600 nm was read (Spectramax Plus 384) for 3 min to establish a baseline. The reaction was then initiated by addition of 10 µl of 1.625 mM coenzyme Q_1 and the decrease in absorbance as a result of reduction of DCPIP was recorded every 15 s for 5 min.

6.3.3.3 Complex III (ubiquinol:cytochrome c oxidoreductase) activity

Measurement of CIII activity was done according to Spinazzi et al. (2012) with a few modifications. Briefly, the mitochondrial suspensions were diluted to 1 mg/ml protein with hypotonic buffer and sonicated to disrupt the IMM and liberate the membrane bound enzyme. To each well of a 96-well microplate, 230 µl of assay buffer (pH 7.3), containing 100 mM

potassium phosphate, 1.4 mg/ml BSA, 0.025% Tween-20, 2 mM KCN and 10 µM rotenone, was added. Subsequently, 10 µg of mitochondrial protein (in 10 µl) and 10 µl of 10 mM cyt c were added to each well, and each sample was assayed in parallel with and without 2 µg/ml antimycin A. The reaction was initiated by addition of 4.5 µl of reduced 7 mM decylubiquinone and the absorbance was read at 550 nm (Spectramax Plus 384) for 3 min. CIII activity was calculated by subtracting the antimycin A-insensitive activity from the total activity.

6.3.3.4 Complex IV (cytochrome c oxidase; COX) activity

Measurements of CIV/COX activity followed the method of Spinazzi et al. (2012) with some modifications. Initially, the mitochondrial suspensions were diluted to 1 mg/ml in hypotonic buffer and sonicated to disrupt the IMM thus releasing the membrane bound enzyme. An assay buffer (pH 7.3) containing 62.5 mM potassium phosphate and 250 µM reduced cyt c was made. Subsequently, 180 µl of the assay buffer were added into each well of a 96-well microplate. The reaction was then initiated by addition of 20 µg of mitochondrial protein (for a total assay volume of 200 µl) and the absorbance was read at 550 nm (Spectramax Plus 384) every 15 s for 10 min. A decrease in absorbance is a measure of CIV enzyme activity. To ascertain the specificity of the assay, 7.5 µl of 10 mM of KCN added to the complete reaction mixture with the mitochondria showed no activity.

6.3.4 Gene expression analyses

The gene expression experimental procedures conformed to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Bustin et al. 2009).

6.3.4.1 RNA extraction

Total RNA was extracted from 100-200 mg of liver mitochondrial suspension or whole liver/gill tissue using the Tri-Reagent extraction method (Chomczynski 1993). Frozen tissue/suspension was immediately added to 1.5 ml of Tri-Reagent and mechanically macerated with a homogenizer (VWR, Mississauga, ON). To avoid cross-contamination, the homogenizer was rinsed between samples in the following sequence MilliQ-water → 70% molecular grade ethanol → molecular grade water. Following 5 min incubation at room temperature, 300 µl of chloroform was added to each sample and mixed by vigorous shaking for 15 s. Samples were incubation for 3 min at room temperature and then centrifuged at 12000 × *g* for 15 min at 4 °C. The colourless aqueous phase was transferred to a new 1.5 ml centrifuge tube and 500 µl of molecular grade isopropyl alcohol was added, mixed and incubated at room temperature for 10 min to precipitate the RNA. Samples were centrifuged at 12000 × *g* for 10 min at 4 °C to obtain RNA pellets. The RNA was washed with 500 µl of ice cold 75% molecular grade ethanol by centrifugation at 7600 × *g* for 5 min at 4 °C; residual ethanol was carefully removed and the RNA was dried by exposure to air for 10-15 min.

The RNA pellets were resuspended in 100-200 µl of molecular grade water and stored at -80 °C until further use. Total RNA purity and concentration were measured spectrophotometrically using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). All RNA samples had 260/280 values in the range of 1.95-2.10. A subset of samples (ca. 30%) were analyzed for RNA quality using a BioRad Experion (Mississauga, ON) with RNA Quality Indicator (RQI) values >9.0. To degrade potential genomic DNA, 5 µg of the total RNA was treated with RNase-free DNase (TURBO DNA-free kit, Ambion, Carlsbad, CA) following the manufacturer's instructions. The

DNase-treated total RNA (1 µg) was then reverse-transcribed (RT) to cDNA in 20 µl reactions using BioRad iScript™ Reverse Transcription kit (Hercules, CA) as per manufacturer's instructions and the cDNA was stored at -20 °C until used for quantitative PCR (q-PCR). Reverse transcription-free controls (-RT) were included to ensure the absence of genomic DNA and both RT and -RT samples were diluted 2-fold with molecular grade water.

6.3.4.2 Gene Expression

Target genes were selected based on their roles in energy metabolism and cellular response to the stressors studied, while reference genes were selected based on unchanged expression in control/treated fish from other studies and were validated under my experimental conditions. Thus, the expression levels of genes encoding proteins involved in metal regulation, mitochondrial respiration and energy metabolism (MT-A and MT-B, COX4-1 and COX4-2, AMPKα1) were measured. Primer sets for MT-A, MT-B, COX4-1, and COX4-2 (Table 6.1) were generated using NCBI Primer-Blast software (http://www.ncbi.nlm.nih.gov/tool/primer-blast/) while those for AMPKα1 (Polakof et al. 2011) and 3 reference genes, eukaryotic elongation factor 1 alpha paralog B (EF-1α), structural ribosomal protein S20 (RPS20), eukaryotic translation initiation factor 3 subunit 6 (eIF3) were obtained from the previous literature (Olsvik et al. 2005; Skugor et al. 2008). The qPCR amplification for each sample was performed on the Mastercycler® ep *real*plex thermocycler (Eppendorf, Mississauga, ON) in an 11 µl reaction containing 0.45 µM of the forward and reverse primer, 5 µl of 2× SsoAdvanced Sybr® Green Supermix (Bio-Rad, Hercules, CA), 4 µl of molecular grade water (Lonza, Rockland, ME) and 1 µl of cDNA (diluted 1:25 in molecular grade water). The thermal profile for each reaction was: denaturation at 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s and

Table 6.1: Primer sets (and their annealing temperatures, efficiency, accession numbers) used for amplification of target (MT-A, MT-B, COX4-1, COX4-2, AMPKα1) and reference genes (EF-1α, RPS20, eIF3) in rainbow trout for quantitative real-time PCR (qPCR) expression studies.

Gene	Direction	Primer Sequence (5'→3')	Annealing Temp (°C)	Efficiency	Accession No:
MT-A	Fwd	GGATCCTGCAAGTGCTCCAA	66.1	0.93	X59395
	Rev	CACAAGTCTTGCCCTTGCAC			
MT-B	Fwd	CCTGCAAGTGCTCAAACTGC	66.1	0.95	X59394
	Rev	AACAGCTGGTATCGCAGGTC			
COX4-1	Fwd	TGGGTGGCCGAAGAATCAAA	66.1	0.99	EZ905106
	Rev	TCGTTTGAGCCGTGCTAGTT			
COX4-2	Fwd	TACGGCGATTTCGAGAAGGG	66.1	0.98	EZ906378
	Rev	CACGCAGGCCACCTTCTTA			
AMPKα1	Fwd	CACCATCAAAGAGATCCGAGAG	63.9	1.0	HQ403672
	Rev	TCAAACTTCTCACACACCTCC			
EF-1α	Fwd	TGCCCCTCCAGGATGTCTAC	57.3	0.96	AF321836
	Rev	CACGGCCCACAGGTACTG			
RPS20	Fwd	GCAGACCTTATCCGTGGAGCTA	57.3	0.99	BG936672
	Rev	TGGTGATGCGCAGAGTCTTG			
eIF3	Fwd	GTCGCCGTACCAGCAGGTGATT	57.3	1.04	CX040383
	Rev	CGTGGGCCATCTTCTTCTCGA			

primer-specific annealing temperature (Table 1) for 30 s, denaturation at 95 ^{o}C for 15 s followed by a melt-curve (ramp from 65 to 95 ^{o}C with fluorescence read after 0.5 ^{o}C increments/5s/step) to verify that only one product was amplified.

A no template control (NTC) of molecular grade water was used in the series of reactions to check for potential cross-contamination between samples. Samples were run in duplicate and only considered acceptable when technical replicates were within 0.5 cycles. Samples with replicates having a threshold cycle difference greater than 0.55 were re-run in duplicate. An internal control and NTC were run for all genes assessed in each tissue. Additionally, -RT controls were run for each exposure in each tissue for all of the genes to verify no genomic DNA was present. Reaction conditions were optimized by generating a standard curve with efficiencies and R^2 values of 0.9-1.04 and >0.95, respectively (Table 6.1). Primer efficiencies ($E=10^{(-1/slope)}$) were calculated by analysis of 5-fold serial dilutions of the reference genes and the genes of interest using pooled cDNA except for AMPKα1, that was diluted 2-fold.

The gene expression data were analyzed using qBasePLUS relative quantification framework software (Biogazelle, Belgium). The geNORM application contained within the qBasePLUS software was used to determine the stability of reference genes and normalized the data by comparing expression of target genes to the reference genes. All three reference genes were found to be stable (M<1 and CV<0.5) in all tissues under all conditions and were therefore used to calculate the calibrated normalized relative quantity (CNRQ) of the target gene transcripts (Hellemans et al. 2007). CNRQ values greater than two standard deviation of the group mean were deemed outliers and removed from the data. The $\log_{10}$ CNRQ data were converted to linear and subsequently $\log_2$ transformed.

6.4 Statistical analysis

The data are expressed as means ± SEM, and the level of significance for all statistical tests was $p < 0.05$. Statistical analyses were performed with Statistica version 6.0 (StatSoft Inc., Tulsa, OK). The data were initially tested for normality of distribution (Chi-Square test) and homogeneity of variances (Levene's test) and log transformed when necessary. The effects of temperature, Cu and hypoxia were analyzed via MANOVA followed by post-hoc pairwise comparisons of means using Tukey's HSD test.

6.5 Results

6.5.1 Liver ETS CI-CIV enzyme activities

Warm acclimation decreased the activities of all 4 ETS enzymes in all 4 test conditions. Liver mitochondrial CI enzyme activity (Fig. 6.1a) was similar in warm and cold acclimated control fish; however, the overall effect of acclimation was significant. Cu exposure resulted in a 100% increase in liver mitochondrial CI activity in cold acclimated fish while Cu plus hypoxia increased it in warm acclimated fish relative to the respective controls. Hypoxia did not alter CI activity relative to the controls but it reversed the stimulatory effect of Cu in cold acclimated fish.

In contrast to the effects on CI, warm acclimation decreased liver CII activity in the controls (Fig. 6.1b). Indeed, warm acclimated fish mitochondria had lower CI activities irrespective of the Cu and hypoxia exposure status as evidenced by an overall significant main effect. Cu exposure increased the activity of CII in both the cold and warm acclimated fish liver mitochondria. While hypoxia did not have a significant effect on CII activity relative to controls, it reduced the

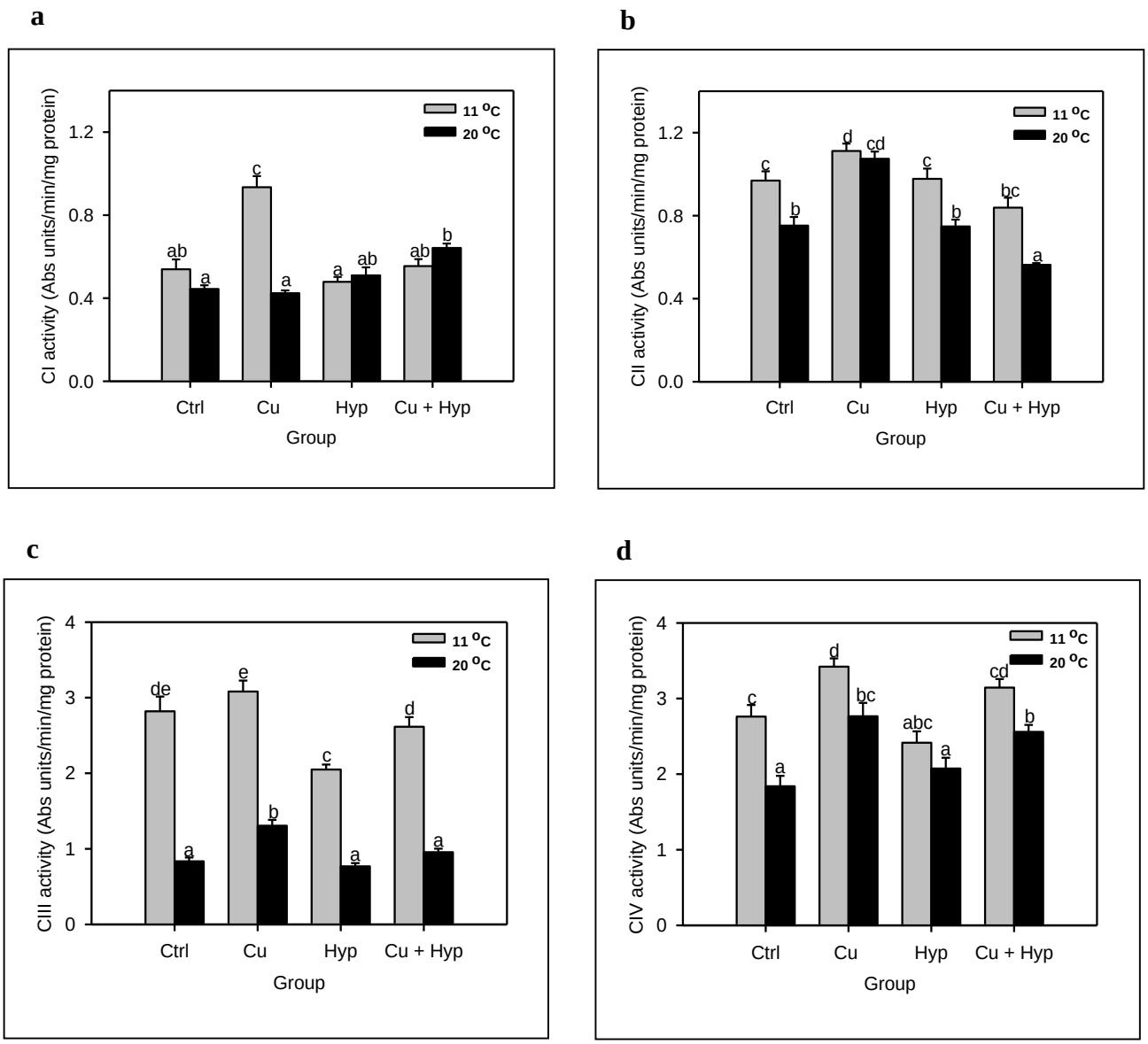

Figure 6.1. Effect of warm acclimation, Cu, hypoxia and Cu plus hypoxia exposure on rainbow trout liver mitochondria ETS complexes I-IV (CI-IV) enzyme activities. a: CI activity; b: CII activity; c: CIII activity; d: CIV activity. The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu+Hyp: 24 h (20 µg/l Cu + hypoxia). Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$, $n = 6$.

stimulatory effect of Cu on this enzyme in cold and warm acclimated fish mitochondria. Cu plus hypoxia reduced CII activity in mitochondria from warm acclimated fish mitochondria relative to the controls as well as the stimulatory effect of Cu in those from cold and warm acclimated fish. Surprisingly, despite the stimulatory effect of Cu alone, the combined treatment indicated that Cu potentiated the inhibitory effect of hypoxia on CII activity.

For CIII enzyme activity, warm acclimation imposed a highly significant inhibitory effect with >70% reduction in activity being observed in mitochondria from warm compared with cold acclimated controls (Fig. 6.1c). Overall mitochondria from warm acclimated fish exhibited lower CIII activity irrespective of the Cu and hypoxia exposure status. Cu exposure stimulated CIII activity in warm without effect in cold acclimated fish liver mitochondria. In contrast, hypoxia reduced liver CIII activity in cold acclimated fish mitochondria and abolished the stimulatory effect of Cu in those of warm acclimated fish. Cu plus hypoxia had no effect on CIII activity relative to the controls but it partially reversed the inhibitory effect of hypoxia.

Lastly, warm acclimation overall significantly inhibited liver CIV enzyme activity (Fig. 6.1d). Exposure to either Cu alone or Cu plus hypoxia increased CIV enzyme activity in warm acclimated fish mitochondria with Cu increasing it in those of cold acclimated fish as well. While hypoxia alone did not cause statistically significant changes in CIV activity relative to the controls, it resulted in lower CIV activity relative to the Cu exposed fish liver mitochondria. Indeed, a comparison of the Cu plus hypoxia exposure with the Cu and hypoxia alone exposures indicated that Cu reversed the inhibitory effect of hypoxia.

6.5.2 Gill ETS CI-CIV enzyme activities

Although warm acclimation did not significantly alter gill CI activity in the controls, it had an overall inhibitory effect (Fig. 6.2a). Cu and hypoxia singly and combined did not alter gill CI activity relative to the controls; however, Cu exposure resulted in lower enzyme activity in warm acclimated fish mitochondria relative to the cold acclimated counterparts. Similar to CI, warm acclimation reduced gill CII activity (Fig. 6.2b). In contrast, Cu exposure did not alter gill CII activity while hypoxia without and with Cu stimulated CII in gill mitochondria from warm acclimated fish. Lastly, both CIII (Fig. 6.2c) and IV (Fig. 6.2d) were recalcitrant to the experimental treatments. Specifically, CIII was not altered by thermal acclimation or exposure to Cu and/or hypoxia while the only significant effect on CIV was its stimulation by hypoxia in warm acclimated fish gill mitochondria relative to the respective control. Overall for gill, Cu and hypoxia did not modulate each other's effects on CI-IV enzyme activities.

6.5.3 Relative expression of energy metabolism and stress/metals response genes

6.5.3.1 Liver mitochondria energy metabolism and stress/metals response genes

Warm acclimation reduced liver mitochondrial COX4-1 gene expression (Fig. 6.3a) relative to cold acclimation irrespective of the status of Cu and/or hypoxia exposure. While exposure to Cu or hypoxia did not alter COX4-1 transcript levels, Cu plus hypoxia significantly reduced its level in cold acclimated fish mitochondria. The overall change in COX4-2 mRNA level (Fig. 6.3b) was inverse that of COX4-1, wherein warm acclimation consistently upregulated COX4-2 expression level relative to cold acclimation regardless of the Cu and/or hypoxia exposure status. Exposure to Cu and hypoxia singly and in combination did not alter COX4-2 gene expression in liver mitochondria.

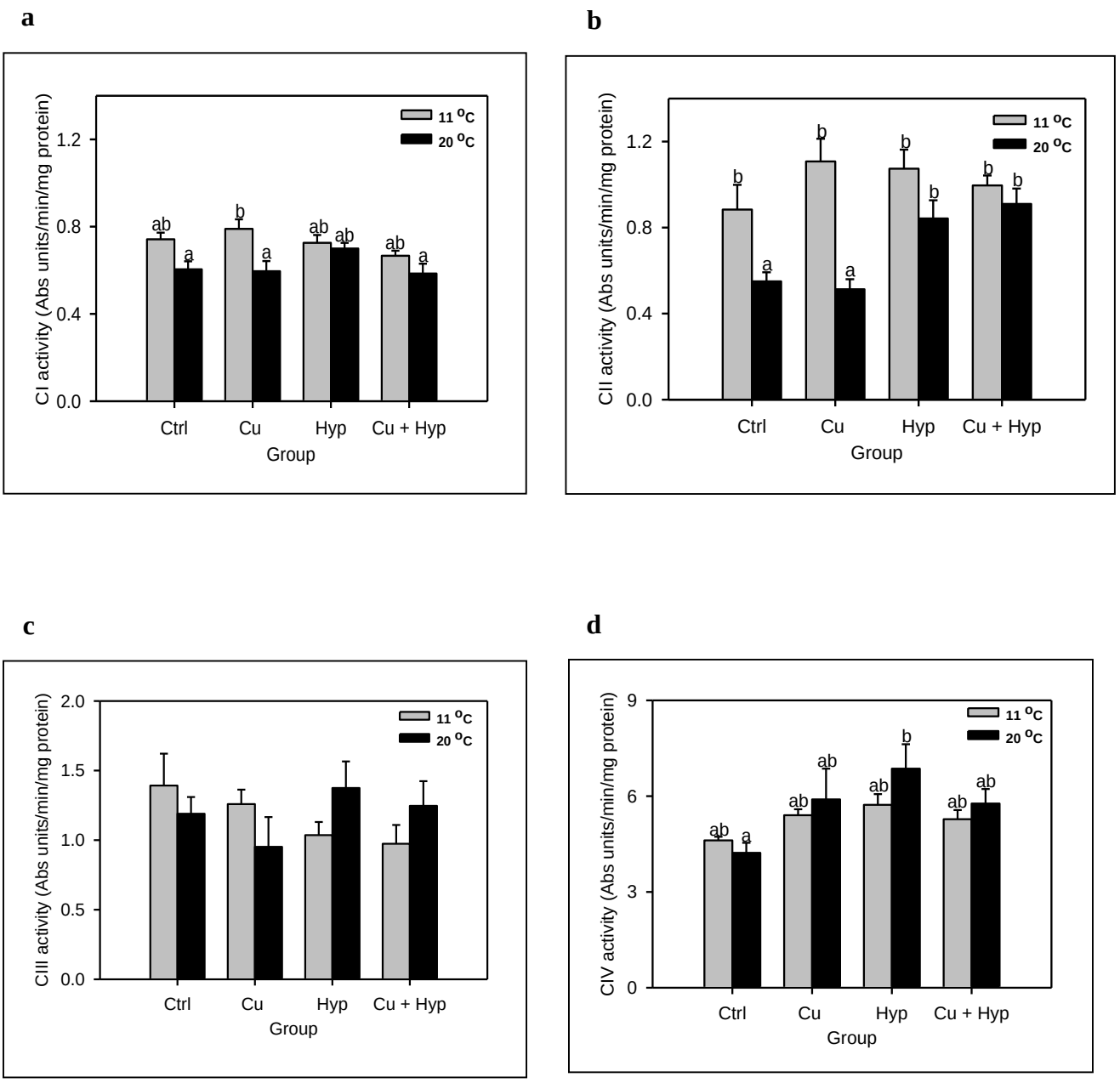

Figure 6.2. Effect of warm acclimation, Cu, hypoxia and Cu plus hypoxia exposure on rainbow trout gill mitochondria ETS complexes I-IV (CI-IV) enzyme activities. a: CI activity; b: CII activity; c: CIII activity; d: CIV activity. The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu+Hyp: 24 h (20 µg/l Cu + hypoxia). Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$, $n = 6$.

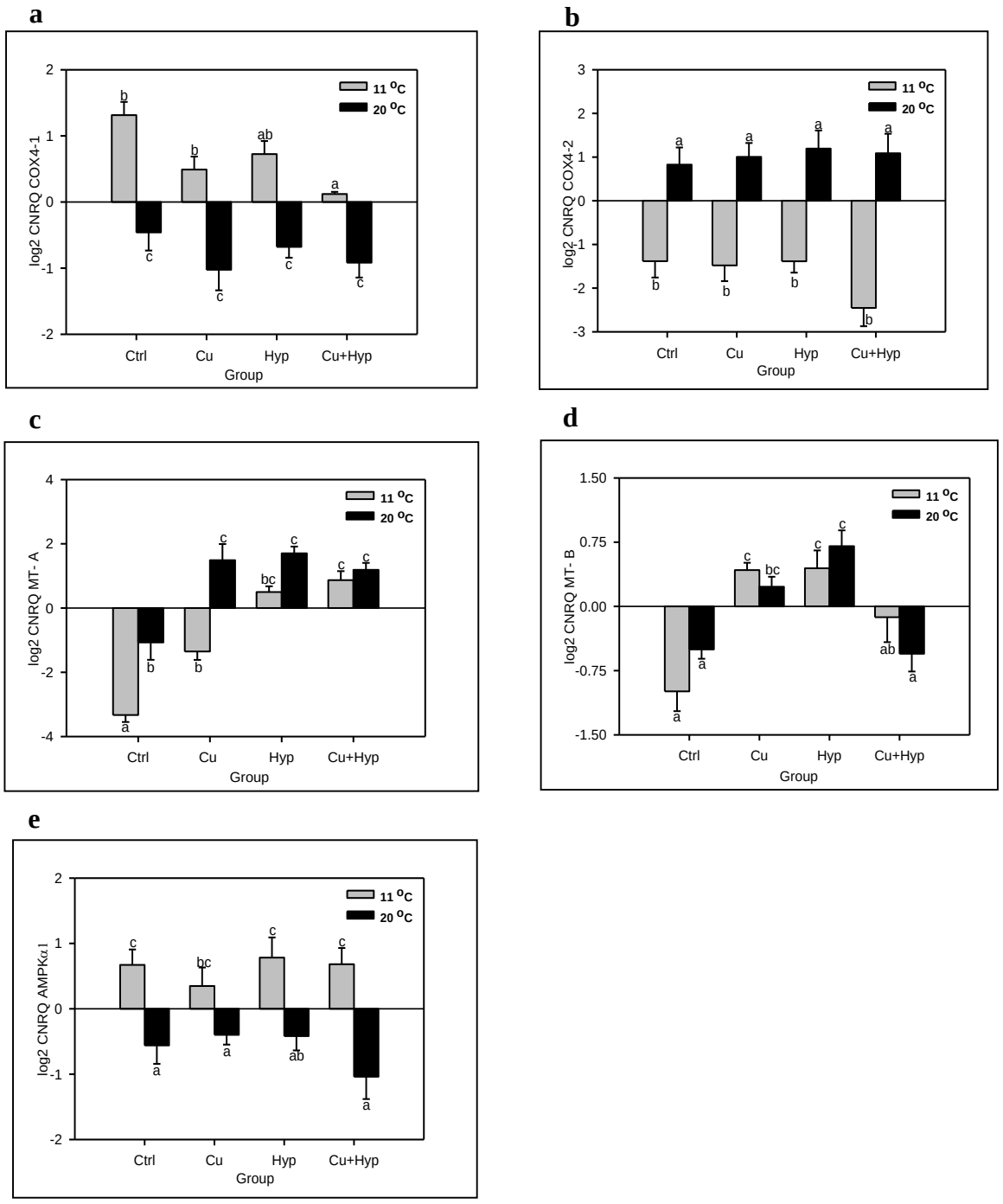

Figure 6.3. Effect of warm acclimation, Cu, hypoxia and Cu plus hypoxia exposure on rainbow trout liver mitochondria relative gene expression of cytochrome c oxidase (COX) subunit 4-1 and 2, metallothionein (MT) A and B, AMP-activated protein kinase α1 (AMPKα1). a: COX4-1; b: COX4-2; c: MT-A; d: MT-B; e: AMPKα1. The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu+Hyp: 24 h (20 µg/l Cu + hypoxia). Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$, $n = 6$.

The expression of the MT-A transcript in liver mitochondria (Fig. 6.3c) was upregulated by warm acclimation with Cu and/or hypoxia enhancing the expression in both cold and warm acclimated fish. Indeed, all the Cu and/or hypoxia exposed fish mitochondria exhibited higher MT-A mRNA levels than their respective controls, while Cu plus hypoxia resulted in higher MT-A mRNA levels compared with Cu exposure alone. Similarly, MT-B gene expression in the mitochondria (Fig. 3d) was increased by Cu and hypoxia in cold and warm acclimated fish. However, unlike MT-A, the levels of MT-B mRNA in liver mitochondria of fish exposed to Cu plus hypoxia were not different from the controls.

Lastly, warm acclimation reduced the gene expression of AMPKα1 in liver mitochondria (Fig. 6.3e) while exposure to Cu, hypoxia and Cu plus hypoxia did not alter its expression level in comparison with the controls.

6.5.3.2 Whole liver energy metabolism and stress/metals response genes

Whole liver responses differed substantially from liver mitochondrial responses. Although the transcript levels in controls were similar in cold and warm acclimated fish livers, warm acclimation overall increased COX4-1 mRNA levels (Fig. 6.4a). Additionally, exposure to hypoxia in cold acclimated fish reduced COX4-1 mRNA levels compared with the control. For COX4-2 (Fig. 6.4b), livers from all warm acclimated fish showed higher mRNA levels compared with the cold acclimated irrespective of the Cu and hypoxia exposure regimes. Neither Cu nor hypoxia alone and combined altered liver COX4-2 mRNA levels compared with the controls; however, COX4-2 mRNA levels were higher in warm acclimated livers exposed to Cu and Cu plus hypoxia compared with their cold acclimated counterparts.

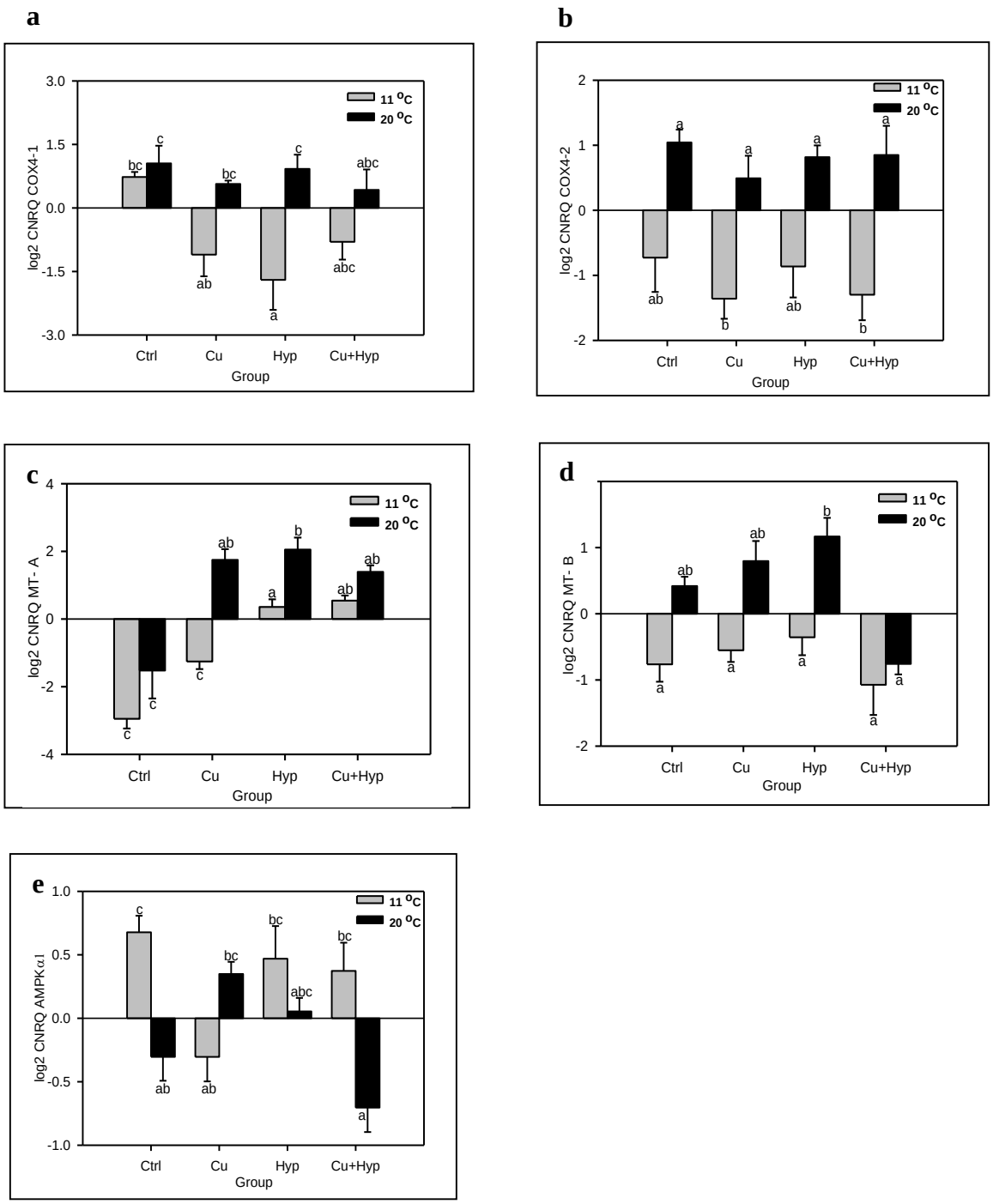

Figure 6.4. Effect of warm acclimation, Cu, hypoxia and Cu plus hypoxia exposure on rainbow trout whole liver relative gene expression of cytochrome c oxidase (COX) subunit 4-1 and 2, metallothionein (MT) A and B, AMP-activated protein kinase α1 (AMPKα1). a: COX4-1; b: COX4-2; c: MT-A; d: MT-B; e: A AMPKα1. The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu+Hyp: 24 h (20 µg/l Cu + hypoxia). Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$, $n = 6$.

While the relative expression levels of MT-A gene in livers of warm and cold acclimated controls were similar, warm acclimation overall increased MT-A mRNA levels (Fig. 6.4c). Exposure to Cu and hypoxia individually and in combination increased MT-A mRNA levels in both cold and warm acclimated fish livers, with a more pronounced effect in the later. For MT-B (Fig. 6.4d), the mRNA levels were overall higher in warm acclimated fish livers. Moreover, Cu plus hypoxia exposure in warm acclimated fish resulted in lower liver MT-B mRNA levels than hypoxia exposure alone.

The expression level of AMPKα1 (Fig. 6.4e) in the liver was lower in warm acclimated compared with cold acclimated fish. Here, exposure to Cu and Cu plus hypoxia reduced AMPKα1 expression in cold and warm acclimated fish relative to the cold control. Additionally, exposure to Cu plus hypoxia resulted in lower liver AMPKα1 expression than Cu exposure alone in warm acclimated fish.

6.5.3.3 Gill energy metabolism and stress/metals response genes

The expression of gill COX4-1 in cold and warm acclimated controls were similar (Fig. 6.5a) and only Cu exposure in warm acclimated fish significantly upregulated the expression of this gene compared with the control. In contrast, gill COX4-2 gene was consistently suppressed by warm acclimation (Fig. 6.5b) wherein the relative mRNA transcript levels were lower in all warm acclimated groups compared with their cold acclimated counterparts. However, within either the warm or cold acclimated fish, exposure to Cu and/or hypoxia did not alter COX4-2 gene transcript levels relative to the respective controls.

The expression level of MT-A (Fig. 6.5c) was overall increased by warm acclimation. Moreover, Cu and hypoxia alone and combined upregulated the MT-A gene in warm and cold acclimated

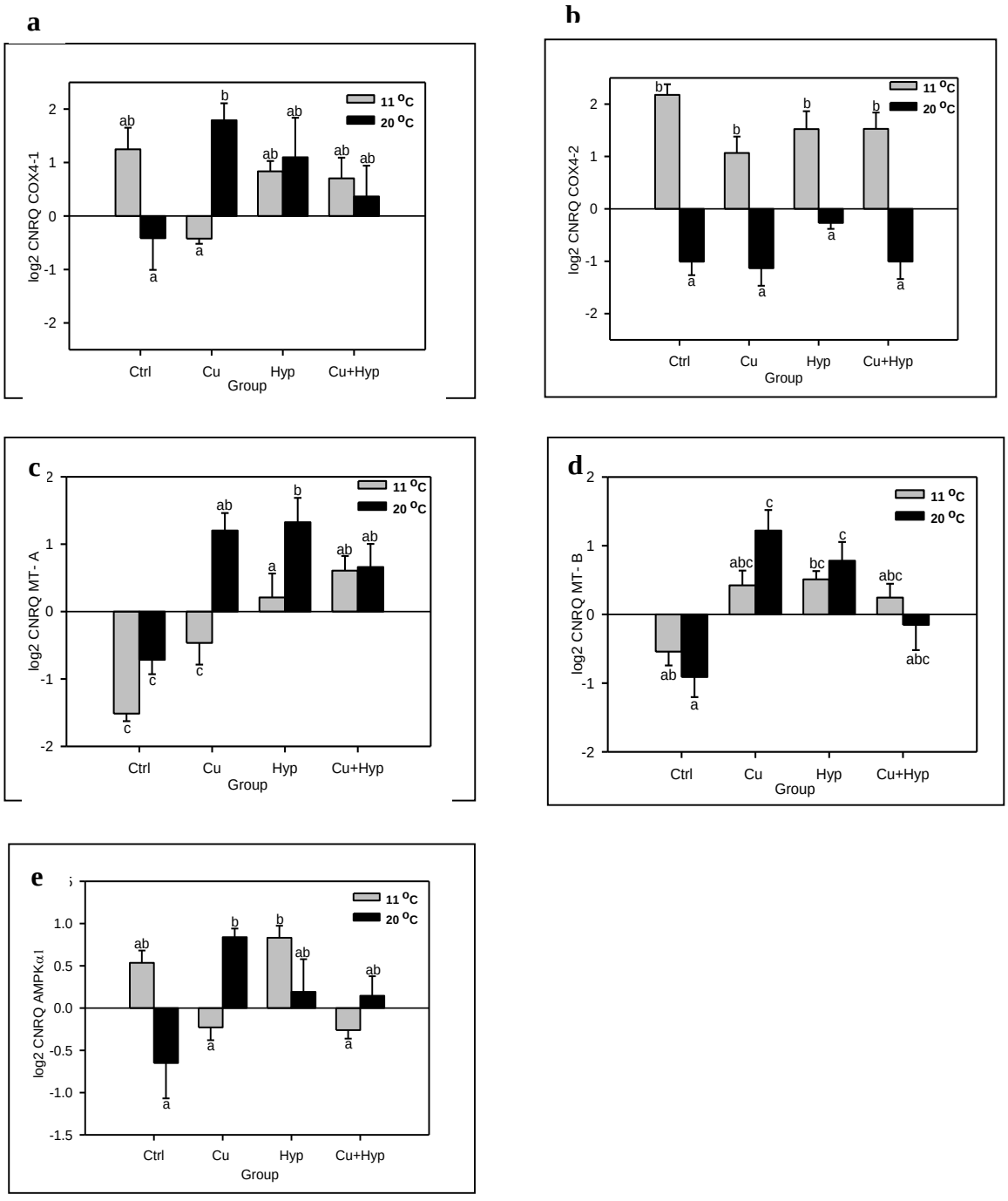

Figure 6.5. Effect of warm acclimation, Cu, hypoxia and Cu plus hypoxia exposure on rainbow trout whole gills relative gene expression of cytochrome c oxidase (COX) subunit 4-1 and 2, metallothionein (MT) A and B, AMP-activated protein kinase (AMPKα1). a: COX4-1; b: COX4-2; c: MT-A; d: MT-B; e: AMPKα1. The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu+Hyp: 24 h (20 µg/l Cu + hypoxia). Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$, $n = 6$.

fish gills. While the expression of MT-B gene (Fig. 6.5d) was not altered by warm acclimation, it was upregulated by Cu and hypoxia in warm acclimated fish gills. All of the other treatments did not significantly alter gill MT-B gene expression.

Warm acclimation did not significantly alter gill AMPKα1 mRNA levels (Fig. 6.5e). However, Cu exposure increased AMPKα1 mRNA in warm acclimated fish compared with the control and Cu-exposed cold acclimated fish. Additionally, gills from cold acclimated hypoxia-exposed fish exhibited higher AMPKα1 mRNA levels than their counterparts exposed to Cu and Cu plus hypoxia.

6.5.4 COX4-2/COX4-1 ratio

To test the idea that the relative proportion of COX4-2 to COX4-1 depends on energy demand, I calculated the ratio of COX4-2/COX-1 using real numbers computed from the log2 mRNA levels data (Fig. 6.6). I found that warm acclimation increased COX4-2/COX4-1 ratio in the liver mitochondria (Fig. 6.6a) but reduced it in gills (Fig. 6.6c) irrespective of the Cu and/or hypoxia exposure. Liver COX4 subunits ratio (Fig. 6.6b) was not significantly altered by warm acclimation. Interestingly, hypoxia increased the proportion of COX4-2 to COX4-1 in livers of cold acclimated (control) fish, whereas Cu increased it in gills. It was also apparent that hypoxia reduced the COX4-2/COX-1 ratio enhancing effect of Cu.

6.6 Discussion

My study investigated the functional-biochemical and transcriptional responses imposed by warm acclimation and their modulation by subsequent hypoxia and Cu exposure. I showed that warm acclimation had profound effects on ETS enzyme activities and expression of selected

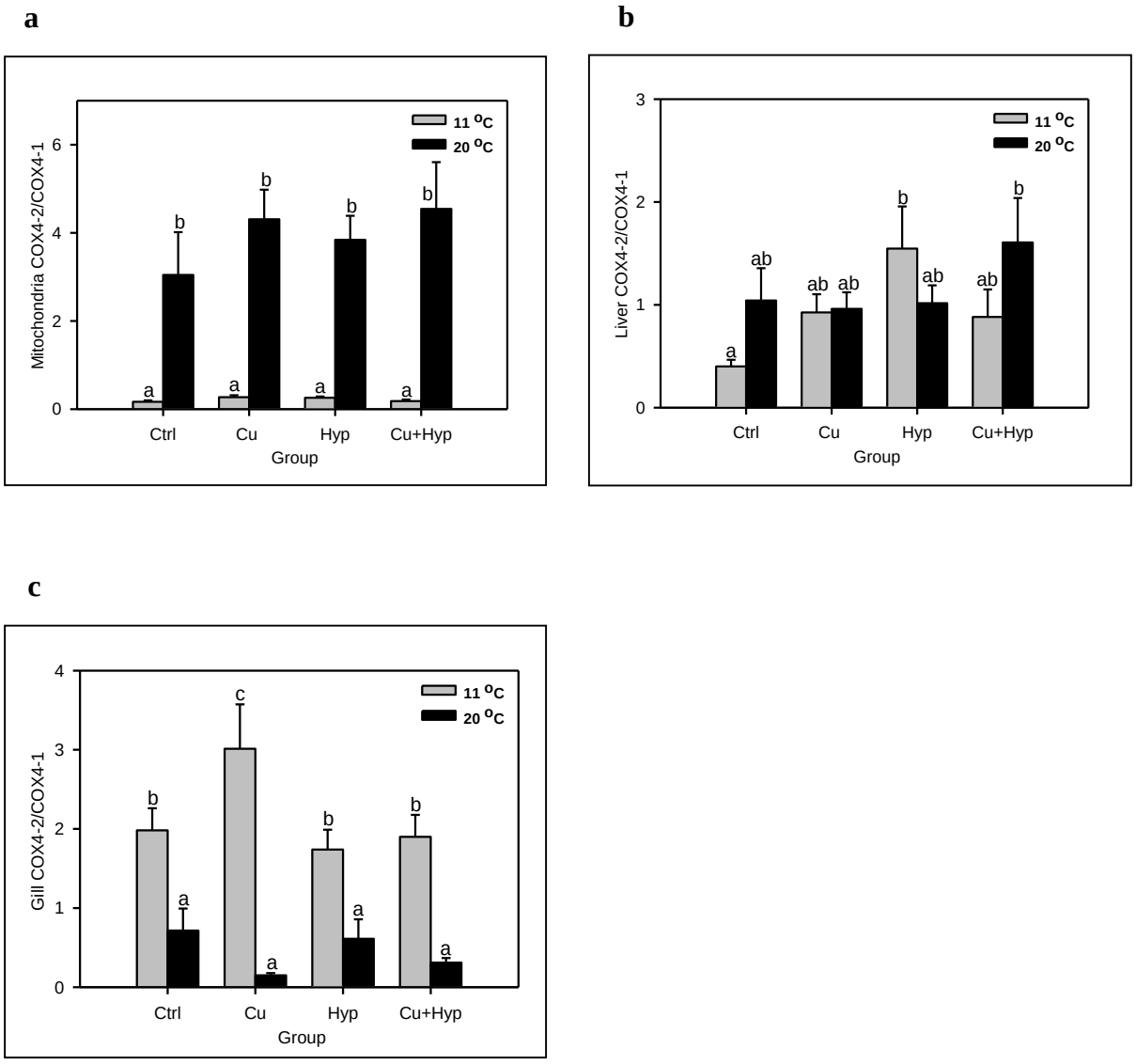

Figure 6.6. Effect of warm acclimation, Cu, hypoxia and Cu plus hypoxia exposure on rainbow trout COX4-2/COX4-1 ratio. a: liver mitochondria; b: whole liver; c: gill. The experimental groups are Ctrl: controls; Cu: 24 h 20 µg/l Cu alone; Hyp: 24 h hypoxia alone; Cu+Hyp: 24 h (20 µg/l Cu + hypoxia). Bars with different letters are significantly different from each other, Tukey's HSD, $p < 0.05$, $n = 6$.

genes encoding for energy metabolism and stress/metal binding proteins, and that hypoxia and Cu acted both independently and interactively to modify these effects.

6.6.1 Effect of warm acclimation, hypoxia and Cu on activities of ETS enzymes

Changes in activities of ETS enzymes, particularly CIV (COX), have been frequently used to gauge the effect of stressors on aerobic energy metabolism (St-Pierre et al. 1998; Lapointe et al. 2011; Oellermann et al. 2012). In this study, warm acclimation differentially reduced the activities of CI-IV in liver and those of CI and II in gills indicating tissue- and enzyme-specificity in the response of the ETS to persistent thermal stress. Interestingly, while liver CIII was clearly the most sensitive to the effects of warm acclimation exhibiting >70% inhibition, branchial CIII, and indeed CI and IV, were recalcitrant to warm acclimation. Similar to my findings, several previous studies have reported decreased activities of ETS enzymes in a variety of tissues from different fish species following warm acclimation (Oellermann et al. 2012; Battersby and Moyes 1998; Kraffe et al. 2007). The reduced activities of ETS enzymes could explain, at least in part, the deceleration of mitochondrial respiration observed in warm acclimated fish by us (Sappal et al. 2015a,b) and others (Guderley and Johnston 1996; Guderley and St-Pierre 2002; Kraffe et al. 2007). The alterations in enzyme activities I observed could have resulted from modulation of affinities of the ETS enzymes for substrates or changes in enzyme conformation and/or quantities. Additionally, temperature-induced changes in membrane phospholipids or fluidity have been shown to modulate ETS activities (Guderley 2004; Kraffe et al. 2007). Surprisingly, although reduction of activities of ETS enzymes with warm acclimation appears to be a recurring finding, increased activity of CIV also has been reported (Cai and Adelman 1990; Seebacher et al. 2005) prompting the suggestion that warm temperature may

induce hypoxic conditions during which high CIV activity would be crucial in sustaining aerobic energy metabolism (Gnaiger et al. 1998).

In my study, Cu at the concentration used either did not alter or stimulated the ETS enzymes. Specifically, CI (cold acclimated), CII, CIII (warm acclimated) and CIV activities in liver increased while no significant changes were observed for gill enzymes. In line with these findings, CI- and II-driven respiration rates were stimulated by low levels of Cu (Sappal et al. 2015b). Consistent with my findings, CIV activity was lower in fathead minnow, *Pimephales promelas* exposed to waterborne Cu at 32 ^{o}C compared with those exposed to the metal at 20 ^{o}C (Lapointe et al. 2011). Overall, on consideration of my findings and those reported in other studies, the effect of Cu on ETS enzyme activities apparently depends on metal concentration, with absence of effect or stimulation at low (Lauer et al. 2012; Hansen et al. 1992; Craig et al. 2007, this study) and inhibition at high (Ransberry et al. 2015; Sokol et al. 1993; Sappal et al. 2014a,b) concentrations. While I did not investigate the mechanisms underlying the Cu-induced changes in enzyme activities, Cu may alter mitochondrial membrane lipid composition via oxidative stress (Sokol et al. 1994; Uriu-Adams and Keen 2005) thereby affecting function of the membrane-bound ETS enzymes. Importantly, one of the ETS complexes, CIV, is a cuproenzyme whose assembly and function depend on Cu (Ghosh et al. 2014; Horn and Barrientos 2008).

Hypoxia induced few tissue- and enzyme-specific effects on ETS enzymes' activities comprising inhibition of liver CIII in cold acclimated fish and stimulation of gill CII and IV in warm acclimated fish. Similar to my findings, hypoxia increased CIV activity in the Pacific oyster, *Crassostrea gigas* gill mitochondria (Sussarellu et al. 2013) while other studies found reduced or no effect of oxygen deprivation on ETS enzyme activities (Colleoni et al. 2013; Galli et al. 2013). Generally, when exposed to brief or moderate levels of hypoxia, fish maintain normal

metabolic rates but during severe hypoxia, mechanisms to reduce oxygen consumption and conserve or produce ATP without requiring oxygen are stimulated (Richards 2009). While the variable and subtle effects of hypoxia I observed suggest that the duration/depth of hypoxia tested was mild, the changes in ETS enzyme activities could have important functional consequences. For example, the increase in CII and CIV activities in gills of warm acclimated fish may facilitate electron supply to ETS thereby sustaining OXPHOS during thermal stress. Moreover, increased CIV activity reflects increase in oxygen affinity of the ETS (Gnaiger et al. 1998) and ensures a favorable gradient for electron flow.

The combined Cu and hypoxia exposure revealed that hypoxia antagonized the stimulatory effect of Cu on liver CI (cold acclimated fish), CIII (warm acclimated fish) and liver CIV in both cold and warm acclimated fish without altering enzyme activities in the gill. Cu also potentiated the inhibitory effect of hypoxia on liver CII activity. These findings are generally consistent with the respiratory activity under the same conditions (Sappal et al. 2015b) in which I reported both antagonistic and additive effects of Cu and hypoxia, and suggest the existence of shared and stressor-specific mechanisms of action.

6.6.2 Expression of energy metabolism and stress/metal responsive genes

To understand the transcriptional underpinnings of warm acclimation, Cu and hypoxia and their interactions, I measured mRNA levels of genes encoding for selected proteins involved in energy metabolism and stress/metals regulation. Specifically, I used COX4-1 and 2 and AMPKα1 to gauge the changes in energy metabolism while MT-A and B were used as metals/general stress indicators.

6.6.2.1 Effect of warm acclimation, hypoxia and Cu on COX4-1 and 2 gene expression

COX, the terminal enzyme complex of the ETS, consists of 13 subunits, of which 3 (COX1, 2 and 3) form the functional (catalytic activity and proton translocation) core of the enzyme. COX subunits 1-3 are encoded by mitochondrial genes while the remaining 10 are regulatory or structural and encoded by nuclear genes (Capaldi 1990; Tsukihara et al. 1995; Barrientos et al. 2002). It is believed that COX4, which exists as 2 isoforms (COX4-1 and COX4-2), regulates the catalytic activity of the enzyme (Huttemann et al. 2001; Li et al. 2006b). In my study, the effects of warm acclimation on the expression of COX4-1 and 2 genes depended on the subunit and tissue. Specifically, warm acclimation suppressed COX4-1 mRNA levels in liver mitochondria while COX4-2 in liver (mitochondria and whole tissue) and gill was upregulated and downregulated, respectively. Overall, these responses resulted in increased and reduced COX4-2/COX4-1 ratio in the mitochondria and gills in warm acclimated fish, respectively. While the switching of COX4-1 to COX4-2 has been described in mammalian cell lines and tissues (Fukuda et al. 2007; Desplanches et al. 2014) in response to hypoxia, I believe my study is the first to show that warm acclimation in an ectotherm induces switching of COX4-1 to COX4-2. COX4 subunit switching is believed to optimize COX oxidative capacity depending on the energy demand, wherein COX4-2 enhances electron transfer and reduces ROS generation (Fukuda et al. 2007; Kocha et al. 2015 and references therein).

Surprisingly, although it has been argued that COX activity corresponds to transcript abundance of the regulatory subunits (Hardewig et al. 1999b; Duggan et al. 2011), I found that the mRNA levels of the two COX4 subunits were either weakly negatively or not correlated with biochemical-functional (CIV activity or respiration) indices. This suggests that quantity of

mRNA of COX4 may not be universally a good indicator of the total COX protein level and by extension function, although protein levels may also not necessarily correlate with function.

The presence of mRNA of COX subunits encoded by the nuclear genome was unexpected in isolated mitochondria. A possible explanation for this finding is co-translational import of proteins encoded by the nuclear genome into the mitochondria, wherein cytosolic nucleus-derived mRNAs dock on mitochondrial surface (Lecuyer et al. 2007; Ahmed and Fisher 2009; Michaud et al. 2014). Indeed, nuclear mRNAs encoding for mitochondrial proteins have been observed in the mitochondrial fraction in a variety of organisms (Michaud et al. 2014 and references therein) and serve to target gene products to the mitochondria. Regardless, the contamination of the mitochondrial fraction by cytosol or endoplasmic reticulum (ER) containing nuclear mRNAs cannot be ruled out.

Cu suppressed COX4-1 gene expression in liver mitochondria and cold acclimated fish gills but upregulated it in warm acclimated fish gill without affecting COX4-2 mRNA levels in either tissue. Based on my best knowledge, this is the first study showing that Cu differentially regulates gene expression of COX4 subunits based on tissue and thermal acclimation status in fish. However, it was shown that Cd did not alter COX4 mRNA level in the eastern oyster, *Crassostrea virginica* hepatopancreas (Ivanina et al. 2011). Additionally, Araya et al. (2012) observed an inconsistent pattern of COX4 mRNA levels in Cu supplemented and un-supplemented healthy human adults. Overall, while additional studies are required to fully characterize the effects of elevated levels of Cu on COX4 gene expression, a clear picture does exist regarding effects of Cu deficiency on COX4 protein and COX activity. Notably, Cu deficiency decrease protein levels of COX subunits (Gybina and Prohaska 2006; Broderius and

Prohaska 2009) resulting in decreased COX activity due to impaired assembly of this cuproenzyme.

COX4-1 and COX4-2 have been proposed to regulate mitochondrial respiration under normoxic and hypoxic conditions, respectively (Fukuda et al. 2007). Specifically, hypoxia diminishes respiratory activity by activating hypoxia inducible factor-1α (HIF-1α), which stimulates transcription of COX4-2, and LON (ATP-dependent protease La), which in turn degrades COX4-1 essentially inducing the switching of COX4-1 subunit to COX4-2. In my study, hypoxia caused downregulation of COX4-1 gene expression in whole liver without altering COX4-2 mRNA levels. Although this suggests that COX4-2 was the predominant isoform during hypoxia, COX4-2/COX4-1 ratio was significantly elevated only in livers of cold acclimated fish. To my best knowledge, this is the first report of hypoxia-induced COX4 subunit switching in fish and contrasts a recent study (Kocha et al. 2015) that did not detect this phenomenon in tissues derived from fish species of varying hypoxia tolerance. My study also highlighted tissue-specific response to hypoxia because the relative proportion of the two COX4 subunits was not altered by this stressor in the gill. A previous study in mammalian cells showed that COX4 mRNA level was unaffected (COX4-1) or increased (COX4-2) by hypoxia (Fukuda et al. 2007) while anoxia exposure in the eastern oyster upregulated COX4 gene expression (Ivanina et al. 2010). Although overall my study and the available data suggest that the effects of hypoxia on COX4-2/COX4-1 ratio depend on the tissue, thermal acclimation status and the hypoxia tolerance of the animal species, further studies are required to better characterize the response of COX4 to stressors.

Combined Cu and hypoxia suppressed COX4-1 gene expression in cold acclimated liver mitochondria implying that COX4-2 was predominant as would be expected under hypoxia.

Moreover, hypoxia did not modify the effects of Cu on either COX4-1 or 2 gene expression, a finding consistent with the lack of interaction of the two stressors on CIV-supported respiration (Sappal et al. 2015b). Overall, while the effects of metals and their interactions with hypoxia on COX4 gene expression are rarely studied, Cd did not alter COX4 mRNA level in eastern oysters under normoxia and anoxia/hypoxia (Ivanina et al. 2010, 2011).

6.6.2.2 Effect of warm acclimation, hypoxia and Cu on AMPKα1 gene expression

The AMPK has been tagged as an energy sensor because it is activated by an increase in the AMP-to-ATP ratio. This protein plays a crucial role in energy regulation, preventing ATP depletion by inhibiting anabolic pathways (ATP consuming) and accelerating ATP-producing catabolic pathways (Hardie et al. 2006; 2012). The three known AMPK subunits (α, β, γ) have been described in fish (Polakof et al. 2011; Jibb and Richards 2008; Stenslokken et al. 2008) but their response to environmental stressors have not been comprehensively studied. Here, I found that warm acclimation suppressed the expression of AMPKα1 gene in liver (mitochondria and whole tissue) but did not alter its transcript levels in the gill. It appears that warm acclimation prompted the maintenance of low levels of AMPKα1 mRNA in the liver, which by extension may result in low AMPK protein levels, a status amenable to anabolic processes including the synthesis of stress defense proteins. Consistent with this notion, Sappal et al. (2015a) reported that warm acclimated fish had higher mitochondrial protein content per gram liver compared with the controls, and speculated that it was in part due to induction of stress defense proteins within the organelle. Lastly, presence of AMPKα1 mRNA in the mitochondria was unexpected. While contamination of the mitochondria with cytosolic or ER mRNA was my first potential explanation, it can be discounted because the AMPKα1 gene expression pattern in the mitochondria was not similar to that in whole liver. However, because it has been reported that

mRNAs commonly associate with organelles where the proteins they encode for are required (Weis et al. 2013), it would not be unreasonable to find AMPKα1 mRNA in the mitochondria.

Whereas Cu did not alter AMPKα1 mRNA levels in liver mitochondria, it suppressed it in cold acclimated whole livers but increased it in warm acclimated gills. Thus, Cu exposure could have increased the AMP-to-ATP ratio in warm acclimated fish gills, with the converse occurring in cold acclimated livers. These differential effects would, if associated with analogous changes in AMPK protein levels, favor catabolic processes including those that generate ATP in warm acclimated fish gills and anabolic processes (e.g., protein synthesis) in the liver. Except for the stimulation of the AMPK pathway *in vitro* by a Cu (II) complex (Filomeni et al. 2011) I am not aware of other studies on the effect of elevated Cu levels on AMPK protein or mRNA levels. However, AMPK was activated by Cu deficiency (Ishida et al. 2013), likely because the resultant impaired assembly and function of the ETS cuproenzyme, COX, led to energy dyshomeostasis.

In my study, hypoxia did not alter AMPKα1 mRNA levels suggesting that the duration/depth of hypoxia used was insufficient to alter AMP-to-ATP ratio. Other studies have nonetheless shown that sufficiently severe hypoxia evoked tissue-specific and generalized AMPK gene expression in goldfish, *Carassius auratus* (Jibb and Richards 2008) and mammalian models (Kudo et al. 1995; Mu et al. 2001; McCullough et al. 2005), respectively. Similarly, the combined Cu and hypoxia did not alter AMPKα1 mRNA levels in comparison with the controls in liver (tissue and mitochondria) and gills. However, in warm acclimated fish, Cu plus hypoxia resulted in lower AMPKα1 expression in liver mitochondria and whole liver relative to the cold acclimated controls and the Cu exposure alone, indicating that warm acclimation and Cu exposure worsened the effects of hypoxia. The downregulation of AMPKα1 gene expression could have resulted

from reduced AMP/ATP ratio, a change that would favor anabolism. It would have been informative to also measure AMPK protein levels and/or activity.

6.6.2.3 Effect of warm acclimation, hypoxia and Cu on MTA and B gene expression

Warm acclimation increased MT-A gene expression in liver (mitochondria and whole tissue) and gill tissue, as well as MT-B gene expression in liver whole tissue, without altering its transcript levels in liver mitochondria or gills. While the MT protein has been localized in the mitochondrial intermembranous space (Ye et al. 2001), the presence of MT-A and B mRNAs in the mitochondria in my study was intriguing. Potential explanations for the presence of MT mRNA in mitochondria are as provided above for COX subunits and AMPK. Regardless, increased MT transcript level in response to heat stress is not unprecedented having been previously observed in aquatic ectotherms (Ivanina et al. 2009; Van Cleef-Toedt et al. 2001). Thermal stress may increase MT mRNA level by direct activation of the metal response element (MRE) in the MT promoter region by heat shock transcription factor or indirectly by activation of antioxidant response element by heat-induced oxidative stress (Tamai et al. 1994; Liu and Thiele 1996). Additionally, ROS may react with MT causing the release of MT-bound Zn ions, which would then activate metal-regulatory transcription factor 1 (MTF-1) resulting in increased transcription of MT genes (Kang 2006; Saydam et al. 2003; Haq et al. 2003).

MTs regulate and/or detoxify metals in biological systems (Amiard et al. 2006; Roesjadi 1996; Klaassen et al. 1999) and their expression is upregulated in response to increased intracellular levels of Zn, Cd and Cu in several tissues of fish (Van Cleef-Toedt et al. 2001; Van Heerden et al. 2004). In my study, the upregulation of MT-A gene expression in liver (mitochondria and whole tissue) and gill tissue, and MT-B gene expression in liver mitochondria and gills is

consistent with this theme. This upregulation occurred in both cold and warm acclimated fish contrasting the finding that heat stress prevented MT gene transcription during Cu exposure in fathead minnow muscle (Lapointe et al. 2011). Moreover, although an earlier study found that MTs were more strongly induced by metal (Cd) than by temperature stress in oysters (Ivanina et al. 2009), in my study warm temperature had the predominant effect probably because exposure (acclimation) period (3 weeks) was much longer than the Cu exposure (24 h) duration, inasmuch as relatively low exposure levels of the metal were used.

Hypoxia upregulated MT-A gene expression in all tissues and that of MT-B in liver mitochondria of cold acclimated fish. Similar to my findings, hypoxia increased MT mRNA levels in gills of the European glass eel, *Anguilla anguilla* (Pierron et al. 2007) and in mammalian tissues (Raleigh et al. 1998; Murphy et al. 1999, 2008). To increase MT transcript levels, hypoxia possibly acted via general stress response wherein increased levels of cortisol would activate the glucocorticoid response element (GRE) in the MT-A promoter (Bury et al. 2008) to regulate MT-A gene expression but not MT-B because the latter lacks a GRE. Alternatively, hypoxia may induce HIF-1α and activate MTF-1 that would act via MRE to promote MT gene expression (Murphy et al. 1999; 2008). Taken together, the induction of MT gene expression by hypoxia (and temperature) observed in my study supports the notion that MTs are indicators of general stress and not just specific biomarkers of metals exposure.

Lastly, exposure to combined Cu and hypoxia upregulated MT-A gene expression but did not alter MT-B transcript levels in both cold and warm acclimated fish. Notably, MT-A mRNA levels were similar for the combined and individual exposures whereas for MT-B, the transcript level was lower in the combined relative to the individual exposures. My results contrast an earlier report in gills of pacu, *Piaractus mesopotamicus* in which combined exposure to Cu and

hypoxia induced MT gene expression more than either stressor individually (Sampaio et al. 2008). However, similar to my study, co-exposure to hypoxia and Cd did not induce a synergistic response on MT mRNA level in glass eels gills (Pierron et al. 2007). Mechanistically, both hypoxia and metals (Cu) likely activated MTF-1 (Murphy et al. 1999, 2008; Uenishi et al. 2006), which then induced MT gene expression. Indeed, several authors have demonstrated that Cu, as well as Cd, mimic effects of hypoxia in mammalian cells and fish (Li et al. 2006a; Martin et al. 2005; Feng et al. 2009; Van Heerden et al. 2004).

6.7 Conclusions

My study highlighted functional-biochemical and transcriptional responses of fish to long-term thermal stress (warm acclimation) and short-term exposures to Cu and hypoxia. Warm acclimation was the most influential factor driving transcriptional and enzymatic responses observed, and its effects were modified differently by Cu and hypoxia. The changes in ETS enzyme activities observed mirrored changes in mitochondrial respiration (Sappal et al. 2015b) under the same experimental conditions in part providing a mechanistic underpinning of altered ETS function. I demonstrated that a select panel of genes encoding proteins involved in energy metabolism and stress response/metals regulation were differentially expressed in fish tissues, with liver being more responsive to environmental stressors than the gill. Overall, the potential co-modulation of gene expression and activity/abundance of energy metabolism and stress proteins by multiple stressors needs to be considered before responses can be conclusively attributed to a single stressor.

CHAPTER 7: GENERAL DISCUSSION AND FUTURE DIRECTIONS

A version of this Chapter is included in a review article:

Sappal R, Fast M, Stevens D and Kamunde C. 2015d. Mitochondrial bioenergetics: a platform to integrate effects of multiple stressors in fish (manuscript in preparation).

7.1 General discussion

Environmental stress in fish imposes physiological constraints that ultimately alter energy homeostasis at the cellular and organism levels. As the primary producers of ATP, mitochondria play a central role in energy homeostasis and as a corollary, stress response. In my thesis research I combined molecular, biochemical-functional, tissue and organismal level measurements to uncover the individual and joint effects of temperature, hypoxia and Cu on mitochondrial function and fish health.

7.1.1 Acute temperature changes exacerbate the effects of Cu on mitochondrial function

Through the first two studies I sought to unveil interactions of acute temperature changes and Cu on mitochondrial bioenergetics. While the notion that mitochondrial energy metabolism might be useful in integrating effects of multiple stressors had been previously proposed (Sokolova et al. 2012), studies pertaining to interactions of temperature and Cu on mitochondrial bioenergetics/ energy homeostasis in fish were lacking prior to my research. Thus, the studies carried out in Chapters 2 and 3 were, to my best knowledge, the first comprehensive analyses of how Cu and temperature modulate each other's effects on mitochondrial function in fish. I showed that Cu exposure causes loss of thermal dependence of maximal mitochondrial respiration and that high temperature sensitizes mitochondria to Cu toxicity. Previous studies had reported increased sensitivity of aquatic invertebrates to metals at high temperatures (Sokolova and Lannig 2008; Altshuler et al. 2011), which, together with my studies suggest that elevated temperature, e.g. due to climate change, would make aquatic organisms more vulnerable to metals exposure.

One of the most interesting findings was that low doses of Cu stimulated proton leak thus increasing mitochondrial inefficiency. Although I used a fish model, this uncoupling property of

Cu could justify investigations on the use of low Cu doses, alone or in combination with other interventions, for weight loss in humans. Regarding the mechanisms of action, I showed that Cu-induced mitochondrial dysfunction resulted from impaired OXPHOS due to inhibition of ETS CI and II, increased membrane permeability through induction of MPTP resulting in the dissipation of MMP, with temperature exacerbating these effects. I also attributed the mitochondrial dysfunction to increase in ROS production which is a widely accepted mode of action of Cu (Pena et al. 1999; Manzl et al. 2003; Krumschnabel et al. 2005).

Another interesting finding (Chapter 3) was that at high temperatures, medium Cu concentrations increased mitochondrial coupling (Fig 3.4A). Though this could be an artifact, other studies have shown that multiple stressors under select conditions induce beneficial/protective effects (Onukwufor et al. 2014, Ivanina et al. 2013) on mitochondrial function. In line with this notion, I additionally found that some combinations of Cu concentrations and high temperature inhibit mitochondrial swelling (Fig. 2.7); the mechanisms underlying this phenomenon currently are not known.

7.1.2 Acute temperature shifts and warm acclimation differentially alter mitochondrial oxidation

In chapter 4 I examined the effects of acute temperature shift, warm acclimation and Cu on mitochondrial respiration supported by CI-CIV. While previous studies had examined effects of acute temperature changes or acclimation on a subset of the ETS segments (Iftikar and Hickey 2013, Strobel et al. 2013) my study was unique in that the respiratory activities of all four of the ETS complexes were measured in one run (Fig. 4.1). I found that for all the complexes, acute temperature rise consistently stimulated while warm acclimation consistently inhibited maximal

respiration rates in agreement with previous studies (Bouchard and Guderley, 2003; Kraffe et al., 2007), albeit on a limited number of ETS complexes. However, the effect of warm acclimation on maximal respiration depended on the ETS complex with the order of inhibition being CI > CII > CIII > CIV. Worthy of note is that concomitant with the inhibition of maximal respiration, basal respiration rates via all the four complexes were stimulated by warm acclimation in a rank order inverse (i.e., CIV > CIII > CII > CI) that of maximal respiration.

7.1.2.1 Warm acclimation, Cu and HRO act additively to impair ETS respiratory activity

Individually, thermal stress, HRO and metals impact energy metabolism (Kraffe et al 2007, Kurochkin et al. 2009, McBryan et al. 2013, Collins et al. 2010), but very few studies have probed the mechanisms and consequences of their interactions on mitochondrial bioenergetics. I tested the hypothesis that these stressors act additively to impair mitochondrial function. While a few previous studies have reported increased toxicity to multiple stressors in fish (Lapointe et al. 2011; Mustafa et al 2012) mine was the first to investigate interactions of warm acclimation with HRO and Cu on mitochondrial respiration in fish. In my study, warm acclimation increased the sensitivity of mitochondria to HRO and Cu and reduced mitochondrial efficiency; however, I found no evidence of cross-tolerance as previously reported for hypoxia and temperature (Rees et al. 2001, Burleson and Silva 2011).

7.1.3 Warm acclimation alters effects and interactions of acute temperature change, hypoxia and Cu

In Chapter 5, I measured the effects and interactions of warm acclimation, hypoxia and Cu at multiple levels of biological organization following *in vivo* exposures to these stressors to gain knowledge on their impact on mitochondrial function, stress response and organismal energy

status. This study revealed very subtle and mostly antagonistic effects among the stressors likely because the stressors were tested at environmentally realistic levels on live fish as opposed to the *in vitro* exposures in Chapter 4. In this study I also uncovered an anterior-distal dichotomy in the sensitivity of the ETS to the stressors tested. While it had previously been reported that ETS enzymes have different thermal sensitivities (Lemieux et al. 2010; Pichaud et al. 2010; Oellermann et al. 2012) I showed that hypoxia and Cu also affect ETS segments differently.

7.1.4 Biochemical and transcriptional responses induced by warm acclimation, hypoxia and Cu exposure

In Chapters 4 and 5 I studied how both acute and persistent temperature changes modify energy metabolism and response of mitochondria/fish to added stressors. Chapter 6 explored the biochemical and transcriptional mechanisms behind those responses. Previous studies have used ETS enzyme activity measurements to study responses of multiple stressors on aerobic energy metabolism (St-Pierre et al. 1998; Lapointe et al. 2011; Oellermann et al. 2012). Moreover, individually these stressors (warm acclimation, hypoxia, Cu) are known to modify ETS enzyme activities (Kraffe et al. 2007; Sussarellu et al. 2013; Ransberry et al. 2015) but a comprehensive analysis of their interactions on energy metabolism and stress response was lacking. My study revealed that changes in ETS enzyme activities mirrored the changes observed in respiration in the previous study under the same conditions. Furthermore, I showed that the liver was more sensitive to environmental stressors than gills, probably because of its higher metabolic capacity.

The most interesting finding of this study was that warm acclimation and Cu induced switching of COX4-1 subunit to COX4-2. My study is the first to show that warm acclimation and Cu cause tissue-specific COX4 subunit switching, a phenomenon that has previously been only

associated with hypoxia exposure in mammalian cells and tissues (Fukuda et al. 2007; Desplanches et al. 2014).

Lastly, I confirmed that MT gene expression is not only upregulated in response to metals exposure (Van Cleef-Toedt et al. 2001; Van Heerden et al. 2004) but also in response to thermal stress (Van Cleef-Toedt et al. 2001; Ivanina et al. 2009) and hypoxia (Pierron et al. 2007). Thus MT gene expression can be used for exploring multiple stressor interactive responses.

7.2 Future directions

My thesis highlighted the potential of mitochondrial bioenergetics as a platform for investigating interactions of multiple environmental stressors and validated a sequential inhibition and activation protocol to measure activity of multiple ETS segments simultaneously, thus saving on resources and generating detailed information in a short time. To solidify this theme, future studies should assess the value of mitochondrial bioenergetics in integrating effects of combinations of other stressors. A prerequisite for studying interactive effects is a clear understanding of the individual effects of the stressors in question. In this regard, there is a need to improve our knowledge base on the effects of Cu on mitochondrial function, starting with uncovering the mechanisms of its uptake into these organelles. Specifically, the use of pharmacological and genomic approaches could determine if Cu utilizes mitochondrial channels for other ions/molecules or Cu-specific pathways to permeate the inner mitochondrial membrane. This would open doors for in-depth studies on the metallation of two key mitochondrial proteins, COX and SOD1, and the effect of environmental stress factors on this process that imparts functionality to these proteins that are critical for aerobic respiration.

Although I investigated the effects and interactions of temperature, hypoxia and Cu at different levels of biological organization, time constraints did not permit studies using cells. Therefore future studies with primary hepatocytes and gill cells would be useful to identify the cellular pathways contributing to mitochondrial bioenergetic responses. Moreover, a comparative approach using fish of different thermal, hypoxia and metal tolerance would provide a more holistic picture and permit the generation of a common theme to describe and model the interactive effects. Ultimately, it would be valuable to incorporate field studies wherein the predictive or monitoring value of biomarkers of effects of multiple stressors identified in laboratory studies would be tested under real world situations.

In my thesis research, I performed targeted analysis of expression of genes encoding proteins of known functions. While I did generate useful and novel information on the interactive effects of temperature, hypoxia and Cu, future studies should combine transcriptomics with proteomics and metabolomics for more holistic and mechanistic understanding of responses evoked by multiple stressors. At the minimum, transcriptional studies should be backed with functional assessments and for energy metabolism the expression of both nuclear and mitochondrial genes should be studied concurrently. Importantly, it has recently been shown that similar to nDNA, mtDNA undergoes epigenetic modification (Iacobazzi et al. 2013). This affords a potentially highly productive ground for discovery of novel biomarkers of effects and interactions of stressors on mitochondrial function and the crosstalk between nDNA and mtDNA epigenetics. Lastly, I was intrigued to find transcripts of proteins encoded by the nuclear genome in the mitochondrial fraction. While I could not rule out contamination of my mitochondrial samples with cytosolic components such as the ER, co-translational import of proteins encoded by the nDNA into mitochondria can result in association of their mRNAs with mitochondria (Lecuyer et al. 2007;

Ahmed and Fisher 2009; Michaud et al. 2014). Clearly, it would be valuable to investigate this phenomenon in future, first to rule out contamination and second, to assess if and how environmental stress alters the import of proteins into the mitochondria.

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